UNIVERSITY OF LONDON THESIS

Degree ~~PhD~~ MD Year 2007 Name of Author ANTHONY HOWARD FELIX MACQUILLAN

THE VARIANCE OF NERVE AXON TO MUSCLE FIBRE RATIO AND ITS EFFECT ON OUTCOME IN FUNCTIONAL MUSCLE TRANSFER

Anthony MacQuillan MB BS, MRCS(G)

A thesis submitted in accordance with the requirements for the examination for the degree of Doctor of Medicine (MD)
University of London

March 2006

RAFT Institute of Plastic Surgery
Mount Vernon Hospital
Northwood
Middlesex
HA6 2RN

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ACKNOWLEDGEMENTS

My sincere thanks to all those who have contributed to this work directly or indirectly through their support of, or work in the RAFT Institute of Plastic Surgery. To Francis Daley, Liz Clayton, Dr. Ros King, Jane and Michelle all of whom contributed greatly to this project and to Sandra Shurey who has the most patience of anyone I know. To Fulvio Ursa-Baiarda whose help and enthusiasm has been fantastic, I suspect that he is the only person on the planet who will want to read this and whose debt I will repay when he comes to writing up!

This work would have been but a figment of my imagination without Mr. Addie Grobbelaar who is the foundation on which this project is built, whose support is immeasurable and who I am proud to count amongst my friends.

Finally, to the Burns and Plastic Surgery Department at Mount Vernon Hospital, I have been privileged to work in one of the cradles of Plastic Surgery and encountered giants in their field. May its spirit and legacy live on forever.

This work is dedicated to my gorgeous Louise,
my parents and family.

ABSTRACT

THE VARIANCE OF NERVE AXON TO MUSCLE FIBRE RATIO AND ITS EFFECT ON OUTCOME IN FUNCTIONAL MUSCLE TRANSFER

Anthony MacQuillan

The results of functional muscle transfer for the treatment of facial palsy are varied. Surgical technique in such cases remains constant with only the selected ramus of the buccal branch of the facial nerve changing. Differing sized branches of the facial nerve in the rabbit were used to reinnervate a constant sized muscle transfer to see if this might explain the spectrum of clinical results seen and additionally provide some insight into the phenomenon of "late onset tightening" seen in some cases.

Peripheral limb reconstruction using functional muscle transfer following injury or tumour resection has been widely reported in the literature. The results of such procedures often fail to deliver the physiological strength that might be hoped for in relation to the size of the transferred muscle. Differing sized pure motor nerves were used to reinnervate a constant sized muscle transfer to see if functional results could be improved in an experimental model analogous to peripheral limb reconstruction.

The rectus femoris muscle in the New Zealand White rabbit was used as a standardised muscle transfer for investigation into how the reinnervating axonal load affects outcome, defined in terms of physiological force developed by the muscle post-operatively, looking at both the central and peripheral nervous systems. Corroboratory investigations were also undertaken to determine the reinnervating characteristics of the nerves studied and those of reinnervated muscle.

Index

Chapter 1

Introduction

1.1 Background

Free functional muscle transfer has become the procedure of choice, when attempting to recreate deficient muscle action, since it was first introduced in 1973 [1]. Whilst much success has been reported using this technique persistent problems have been identified with such operations, problems that have yet to be fully resolved: Bulkiness, under activity, spasticity (or over activity) of the muscle transfer are recurring difficulties throughout the development of this branch of surgery that the clinician has had to face and that are still ongoing problems.

Muscle transfers undertaken for peripheral limb reconstruction are vulnerable to relative weakness in terms of force output, and flaps used in facial reanimation procedures are prone to both under activity and so called over activity (where the muscle graft initially develops pleasing motion but progresses to excessive pull causing distortion of the features). The failure to demonstrate any return of function post transfer (despite the presence of a "healthy" vascularised muscle graft) is a final outcome that can also affect both types of procedure.

The aim of this work was to investigate why peripheral transfers do not become over active when this is a common occurrence in their centrally reinnervated cousins, to investigate whether the results seen in facial reanimation surgery may potentially be explained by the degree of muscular reinnervation and to see how peripheral and cranial nerves differ in their characteristics as donor nerves used to reinnervate muscle transfers.

An experimental design was chosen that paralleled the aforementioned clinical procedures, and allowed the development of both a peripheral and cranial nerve model. The rabbit rectus femoris muscle was chosen as the muscle transfer model as previous experience with this had been widely reported in the literature, and it was suitable for both peripheral and cranial nerve transplants.

1.2 Clinical Work

1.2.1 Facial reanimation

The first case of facial reanimation using microneurovascular techniques was that undertaken by Harii in 1976 using gracilis as the reanimating muscle and the deep temporal motor branch of the trigeminal nerve as the donor nerve [2]. His results demonstrated the value of the technique, though by using the trigeminal nerve as the motor nerve for the muscle, the result was robbed of spontaneity. The use of cross facial nerve grafts to restore function to the paralysed hemi-face had been attempted by the like of Anderl, though ultimately this technique failed to prove successful. It did however offer the chance for transferred muscles to move with spontaneous emotion [3] [4] [5]. The first use of a cross facial nerve graft to give spontaneous emotion to an imported muscle did so however using local muscle transfers or non-vascularised transplants [6]. Increasing experience with the operating microscope soon saw vascularised muscle transfers being coupled with cross facial nerve grafts and with this, the possibility of spontaneous emotion being restored to the paralysed face became reality. Harii out lined several basic tenets for the use of vascularised muscle transfers in his initial paper [2]. These guidelines formed the basis for the selection of suitable donor muscle units, and how the transferred tissue must be utilised at its destination. To be suitable for use, the candidate muscle unit had to be removable from its original site without any residual functional deficit, and had to have a nerve and vessels suitable for microsuturing[1]. Furthermore the muscle shape had to correlate with its proposed application, and once

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[1] Harii noted that not all muscles had a single neurovascular pedicle, and quoted work by Hollinhead and Markee which demonstrated that many muscle has multiple, segmental innervation, unsuitable for free functional muscle transfer. Hollinshead, W.H. and J.E. Markee, *The multiple innervation of limb muscle in man.* J Bone Jt Surg (Br), 1946. **28**: p. 721.. These observations were pre-empting the work of Mathes and Nahai who subsequently classified all muscles according to their vascular anatomy and thus suitability for employment as a muscle flap, either in part or as a whole. Mathes, S.J. and F. Nahai, *Classification of the vascular anatomy of muscles: experimental and clinical correlation.* Plastic and Reconstructive Surgery, 1981. **67**: p. 177-187. According to their classification system muscles belonging to Type I, II and V are primarily chosen for muscle transplantation . Doi, K., et al., *Basic science behind functioning free muscle transplantation.* Clinics in Plastic Surgery, 2002. **29**: p. 483-495.

transferred to the recipient site had to be inset under proper tension (although no elaboration upon what represented the proper tension was made!). With continued refinement of technique, many additions and modifications to these guidelines have been made (summarised elegantly by Doi [9]), though it was with these parameters in mind the search for donor muscle units for use in facial reanimation began.

1.2.2 Donor muscle units in facial reanimation

Many muscles have been advocated for use in facial reanimation based on favourable anatomic features, vascular reliability, ease of harvest and minimal donor site morbidity. Additionally, those favouring a single stage operation have advocated muscles with a suitably long neurovascular pedicle.

Anatomic suitability of a donor muscle requires the graft to be thin, so as not to cause too much soft tissue bulk in the cheek, with a fibre direction that is similar to that of the indigenous facial musculature. Furthermore, the amount of excursion achieved by the muscle during contraction (a function of fibre arrangement – parallel or pennate) is important, with a dynamic range of approximately 2.5cms being quoted as the optimum length of shortening [10]. As most unilateral facial palsy procedures employ a cross facial nerve graft the length of neural pedicle is not of great importance, and similarly the facial artery and vein can be tailored to suit the flap rather than the former dictating the choice of the latter. Donor site morbidity is reported as being low with most flaps, with a high degree of skill being required to harvest any muscle. The main exceptions to the above tenets are in Moebius Syndrome, and patients where a single stage procedure is performed (connecting the donor muscle nerve directly to the contralateral facial nerve without the use of a cross facial nerve graft). Here the need for a longer neural pedicle (to reach the masseteric branch V in the case of Moebius patients or the contralateral VII nerve in single stage procedures), limits the choice of donor units to either the gracilis or latissimus dorsi muscle in most cases.

The first reported case of facial reanimation used gracilis as the transplanted motor unit, with its favourable neurovascular pedicle being cited as one of the main benefits of muscle [2]. Subsequent experience with the muscle demonstrated that when transferred

as a whole the bulk and range of excursion of the muscle presented somewhat of a problem with revision procedures having to be undertaken to correct displacement of facial landmarks by the muscle (both at rest and during motion) [11]. Suitable intramuscular neurovascular morphology (longitudinal nerve and vessel patterns) meant that the flap had the potential for modification, and reduction of both muscle size and excursion distance was achieved by defining the fasicular territories of the muscle and undertaking single fascicle muscle transfers based on the dominant vascular pedicle and a portion of the gracilis innervated by a single nerve fascicle [12] [10]. This method produced favourable results and is now the accepted manner in which to modify the muscle for use in facial reanimation [13] [14] [15] [16] [17] [18].

Subsequent to the initial use of gracilis, extensor digitorum brevis was tried as a muscle donor unit in both single and two stage procedures for facial reanimation [19, 20] [21] [22]. The potential benefits of this particular muscle were the presence of a long, predicable neurovascular bundle and reduced tissue bulk (when compared to gracilis). The muscle did not require trimming prior to inset (just as well since studies have shown that its random intramuscular vascular pattern renders it unsuitable to such modification [10]) and its tendonous insertion provided a ready source of fixation to the zygomatic arch. Most authors however reported poor results with the muscle and it was eventually abandoned in favour of other donor units.

The recognition that significant reduction in muscle bulk occurred following transfer together with a reduction in functional capacity (as had been demonstrated in the experimental setting and confirmed clinically with use of extensor digitorum brevis) prompted some clinicians to search for larger donor units. The longitudinal intramuscular neurovascular pattern seen with latissimus dorsi suggested suitability of the muscle for segmental transfer [23]. Several authors have advocated use of this muscle, particularly in single stage procedures or for Moebius patients, referring to reliability of the neurovascular pedicle, the multidirectional pull achievable when using multiple leaves of muscle and on the good excursion achieved (though others have cautioned against its use due to observed failure in this last category) [24]) [22] [25] [26] [27] [28].

One of the criticisms of both gracilis and latissimus dorsi is the need to trim the muscle to avoid problems of bulkiness in the cheek pouch, and the subsequent increased risk of

post operative haematoma formation. A muscle which offered sufficient bulk (but not so much as to require modification), excursion and similarity in terms of fibre direction with respect to the facial musculature, was pectoralis minor, first described by Harrison in 1984 [10] [29] [30]. The flat fan shaped muscle leant itself to placement within the cheek pocket without the need for modification, and possessed a dual source of innervation, potentially allowing for separate ocular sphincter function [30]. Of the available muscles this perhaps offered the least donor site morbidity [31].

Gracilis, pectoralis minor and latissimus dorsi continue to represent the most commonly used muscles in facial reanimation procedures. Other donor muscles have been reported in the literature and include the rectus femoris [32] [33], rectus abdominis [34] [35], abductor hallucis [36] and serratus anterior muscles [16] [37].

There are few long term, large series publications on facial reanimation. Those published include the series of Terzis, O'Brien, Chuang, Kumar and Harrison [26] [16] [18] [14] [38]. The variability of results from such procedures is demonstrated in these series, with most authors reporting a significant minority of patients requiring some revisional surgery. Of note is the 12% haematoma rate reported by O'Brien in his series of trimmed gracilis flaps, Kumar reporting a 4% rate for the same complication. Terzis reported an 80% fair to excellent outcome rate (though 35% of patients required a supplemental temploralis transfer to achieve adequate commissural movement), Kumar approximately 90% good or excellent (for both 1 and 2 stage procedures), and O'Brien 51% fair to good in their respective series of facial reanimation procedures, results being graded according to post operative movement parameters. These figures whilst obviously not directly comparable (as each author graded results using differing parameters) demonstrate that between 10 to 49% of patients undergoing facial reanimation using functional muscle transplants achieve inadequate or no movement post-operatively.

Amongst those included in the group of patients with good post-operative movement are a sub-group who demonstrate pleasing initial movement but go on to develop over stimulation of the muscle transplant. Varying rates are again given for the development of this phenomenon, Chuang quoting an incidence of between 20 – 30%, Harrison 33% and O'Brien stating that 59% of patients required a "muscle release" procedure for post

operative tethering. Kumar describes post operative muscle tethering in 13% of 2 stage procedures and 10% of single stage procedures, though attributes this to scar formation between dermis and the raw muscle surface of the transferred flap. The tethering described by Kumar seems similar to the dynamic tightening reported by the aforementioned authors.

Kumar's data is the only series to directly compare results between 1 and 2 stage transfers using the contralateral VII nerve as motor source and notes little difference between the 2 types of reanimating procedure save for improved static position in the classic 2 stage operation, and earlier onset of movement seen with the single stage (this together with the lack of sural nerve graft donor site morbidity being the reasons cited by the author as to his preference for the latter type of operation). Single stage procedures undertaken using the ipsilateral facial nerve stump as the source of reinnervation are felt by some to provide the optimum results from facial reanimation [16] [39], however other authors have experienced increased rates of muscle overactivity or contracture when using the ipsilateral facial nerve [40]. Contrastingly Moebius patients, in whom the masseteric branch of Vc nerve is used to reinnervate a functional muscle transfer, do not appear to develop progressive muscle contractures.

1.2.3 Peripheral limb reconstruction

The first clinical case of peripheral limb reconstruction reported was in 1976 [1] using the pectoralis major muscle to recreate forearm flexor function following Volkmann's ischaemic contracture. In the discussion of the paper, several points were raised regarding the operation including the fact that the original motor branch to the muscle which had lost function should be used to reinnervate the transferred flap, and that the transfer should include enough fibres to compensate for the function of the defective muscle and be sufficiently long to provide adequate tension after anastomosis.

The knowledge gained from this work and experimental experience, resulted in wider application of functional muscle transfer in the extremities. Initial reports by O'Brien and Manktelow described similar cases of forearm flexor and extensor reconstruction [41] [42], with subsequent reconstruction of upper arm defects also being reported [43].

Continued refinement of technique and its wider application has lead to the use of functional muscle transfer for limb salvage and sparing surgery following oncological resection, replacing muscles from the quadriceps and hamstrings, to lower limb compartments as well as the shoulder musculature [44] [45].

1.2.4 Donor Muscle Units in Limb Reconstruction

The indications for use of free muscle transfer in the periphery are obviously different to those experienced in the cases of facial reanimation. Similarly the requirements of the donor muscle are different. The range of excursion of the muscle must usually be large – larger at its original site than at that to which it is transferred to, thus ruling out most pennate muscles (with their relatively small range of excursion) as candidates for transfer in this setting. The muscle must be able to generate sufficient force to recreate the function that it is replacing and therefore be of an adequate cross sectional area. The muscle must also have a suitable neurovascular pedicle, preferably with a single neural input for optimisation of force production [46] [9] [47]. In practice these considerations mean that the parallel fibred gracilis, latissimus dorsi and pectoralis major muscles are most often favoured as reconstructive units in this setting (though pectoralis major with its poly neural input is held by some authors in less regard).

Similarly to facial reanimation surgery variable results have been reported with free functional muscle transfer to restore peripheral limb use [48] [49] [41] [43]. The availability of direct functional comparison with the uninjured contralateral side has allowed qualitative estimation of recovery post muscle transfer under certain circumstances (for example force generated by a muscle transplant to restore finger flexion). Published data is however limited, with the reported series being small [49] [43]. The results of these studies demonstrate 30 – 50% recovery of function, when compared to the normal contralateral side, though isolated case reports quote near full recovery for some transfers of smaller muscles [50]. Estimation of recovery in most other series of peripheral limb reconstruction is difficult, as often at least some other co-agonist muscles are still working.

1.3 Experimental Work

Initial experimental work on free muscle transfer with a view to use in clinical plastic surgery utilised non-vascularised muscle transfers [51, 52]. Dependence of the muscle on capillary in growth, inevitable central necrosis and subsequent loss of muscle mass were characteristic features of such transfers [53]. With only limited success achieved by non vascularised transfers both clinically and experimentally, the advent of the operating microscope offered the possibility of vascularised functional muscle transfer [54] [55] [53] [56]

1.3.1 Microvascular studies

The first experimental work using microvascular anastomoses was undertaken in 1970 demonstrating that by 5 months post operation the (canine recutus femoris) muscle structure was near normal and that early central necrosis seen with non vascularised muscle was transfers absent except in cases of anastomotic failure [57]. This finding was further endorsed when vascularised and non-vascularised muscle transfers were directly compared [58] [59] demonstrating microvascular anastomoses maintained superior muscle structure and bulk[2] post transfer. The next step was to examine how circulatory interruption affected muscle transfers.

Ischaemia time was felt to be an important factor governing ultimate muscle recovery with limits of ischaemia being set (arbitrarily) at 90 minutes [57]. However, clamping of

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[2] In his 1978 review paper on (non-vascularised) muscle grafting Carlson. Carlson, B.M., *A review of muscle transplantation in mammals.* Physiology Bohemoslovaca, 1978. **27**: p. 387-400.) comments that at most 2-5% of all fibres in a muscle graft survive, with fibre regeneration occurring in conjunction with capillary in growth. Larger muscle transplants were often noted to have a core of connective tissue representing fibres that had undergone complete ischaemic degeneration prior to being reached by capillary ingress. Although claims of a return of 50% of muscle bulk at 90 days could produce some muscle function, these figures were only achievable in the EDL muscle of the rat, too small a size to be of clinical benefit.

the vascular pedicle to mimic vessel anastomosis for a period of 3 hours was found to produce only minimal changes in the muscle compared to an unoperated control [61]. Further laboratory work using the canine gracilis model showed no deleterious effect on muscle recovery with ischaemia times of up to 4 hours [62, 63]. Flow direction in the transferred muscle was not examined until 1989 [64] a surprisingly lengthy delay given the dates of the pioneering studies, however (perhaps less surprisingly) reversal of flow direction within the muscle resulted in massive damage to the microstructure of the muscle with infarction occurring by 24 hours.

1.3.2 Muscle tension

Tenotomy results in numerous muscular changes that have been reported both at the gross anatomical and molecular levels [65]. These changes include muscle fibre atrophy [66] [67] [68], increased perimyseal and endomyseal connective tissue [69], decreased force generating capacity [70] [67] and decreased serial sarcomere number [71, 72]. It is known that the unloading of a muscle alters its functional ability almost immediately (a 50% reduction in developed force at 24 hours) but that it can regain 100% function (even after 7 days) within 24 hours following the restoration of appropriate tension across the muscle [73]. As postulated by other authors, this is likely to represent some sort of disruption of the excitation-contraction coupling system within the muscle, as the changes occur too quickly to attributable to structural reorganisation [65]. The subtlety of the relationship between resting tension and muscle function is demonstrated by the fact that tenotomy (without tendon repair) leads to a quantitative up regulation in the expression of N-CAM (neural cell adhesion molecule), a marker of fibre denervation in adult skeletal muscle [74] [65], within 24 hours of the insult.

The effects of tenotomy and repair were examined as part of the group of surgical procedures performed by Terzis [75]. The reported effects were interesting as the outcome measure of this study was maximal tetanic force development of the operated muscle. Under the simple conditions of mobilisation and repair of both the origin and insertion of the muscle, a 50% decrease in force was recorded at 14 months post

procedure[3]. This result contrasted dramatically with other work, which failed to demonstrate any deterioration in muscle function after simple tenotomy and repair [76] [73].

Work examining how alteration of the tension under which the muscle is inset has however demonstrated a decrease in functional ability post procedure [76] [77]. An elongation of tendon-muscle apparatus of 4.25% in the rabbit rectus femoris resulted in a tetanic force deficit of 22.5% [76], and an average elongation of approximately 15% (in the soleus-tendo calcaneus apparatus in the cat) a 25% defict [77][4]. In the latter study a decrease in sarcomere number was responsible for the maintenance of overall muscle tendon apparatus length at a constant value. Due to the pennation angle of the muscle fibres, this equated to an approximately 50% reduction in the sarcomere number in each fibre. This resulted in a loss of muscle mass, as commented on by the authors, though sadly the percentage decrease (in mass) was not recorded. In the former paper a decrease in muscle mass was also observed, together with a decreased force production however when adjusted for weight there was no change in force production per gram of muscle.

The relationship between the resting tension set upon a muscle and its ability to perform work is perhaps even more complex for a transferred muscle as the proprioceptive mechanisms for the muscle are no longer intact. It would seem that there is some degree of intrinsic regulation of tension within the muscle, as work by Frey *et al.* demonstrated that division of the patella tendon, together with the motor nerve and simulated ischaemia time of 3 hours resulted in a mere 4% reduction in tetanic force 6 months post procedure. In this experiment only the insertion of the muscle was divided however, and these results differ dramatically when compared to the functional outcomes of muscle transfers (inset into the contra lateral thigh) by the same author, in which only 55% of generated force was recovered compared to a control muscle [78]. It

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[3] This finding was remarkable since it proved to be more of an insult to the functional integrity of the muscle than division and repair of its neurovascular pedicle (which was estimated to cause a 20% force deficit at a similar time point).

[4] Both studies undertook muscle force measurements at approximately 6 months post operatively.

would seem that even if 2 out of 3 of the muscles reference points (origin, insertion and proprioceptive innervation) are disrupted then intrinsic regulation of muscle fibre tension is preserved, though if the muscle is deprived of all 3 sources then at least some of the regulatory ability of the muscle is lost. The reduction of force produced by the muscle (which has been re-inset under its original resting tension) may potentially be explained by a sustained disruption of the excitation-contraction coupling system, as postulated by other authors to be responsible for acute reductions in muscle force following tenotomy [65].

To date there has been no work on how muscle transfers (as opposed to muscles with an intact origin or neural supply) are affected by sequential reductions in resting tension.

1.3.3 Nerve repair

The quality of muscle function seen with differing types of muscle reinnervation was examined by Frey *et al.* where direct nerve suture, nerve implantation (into the muscle belly) and muscular neurotisation were compared [79]. The results of end-to-end nerve repair were similar to that of a previous study [80], giving a 75% recovery of generated force. Nerve implantation resulted in a 61% recovery of generated force, and both types of reinnervation produced multiphasic EMG waveforms of almost normal amplitude. Muscular neurotisation resulted in waveforms that were always smaller in amplitude than those of the control. Nerve suture and implantation were considered by the authors to be superior to muscular neurotisation, and concluded that direct end-to-end nerve repair should be affected wherever possible.

Superiority of perineural over epineural repair has not been established [81]. Some authors have advocated that such suture techniques are not in competition, but that there are indications for each. Recommendations include that monofasicular nerves are repaired with an epineural suture, and that outer epineural (rather than group fasicular) suture should be done in the case of an acute, clean, sharply transacted nerve. As virtually all functional muscle transplants are done as elective procedures using motor nerve branches to power them, it is unlikely that perineural repair would give any advantage (as the nerve ends would be cleanly transacted and contain almost

exclusively motor axons)[5]. Indeed sutures placed into the perineurium have increased potential to result in axonal damage.

Although it is widely acknowledged that end-to-end neurorrhaphy is the optimal form of nerve repair [79] [82] [83], certain clinical situations preclude its use. Avoidance of damage to the donor nerve (and thus any deleterious effect on its target organ) whilst effecting reinnervation of an additional muscle, has been examined by use of end-to-side neurorrhaphies. Several studies have demonstrated the efficacy of this technique using histological and nerve conduction/EMG studies [84] [82] [85] [86]. The use of an epineural window to aide axonal sprouting has been advocated by many authors [84] [82] [85] [83], though some have stated that a perineural window is required for optimal results [86] [87]. Direct comparison of the effects of end-to-end and end-to-side neurorrhaphy (with and without epineural window) on the functional recovery of muscle transplants was examined in a study by Grobbelaar [83]. The results demonstrated the ability of an end-to-side neurorrhaphy to adequately reinnervate a muscle transplant, though functional recovery was delayed when compared to direct end-to-end nerve repair[6].

1.3.4 Denervation and reinnervation changes - morphological

Transection of the motor nerve to a muscle is an inevitable consequence of free functional muscle transfer. The effects of denervation and reinnervaton have been the subject of many investigations in an experimental setting [88] [89] [90] [91] [92] [93] [94] [95] [96].

Normal muscle displays a mosaic appearance of fibre types (I, IIa, IIb), due to distribution of the fibres of a single motor unit throughout a region of muscle [97]. All

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[5] One area where fasicular repair is indicated is in segmental muscle transplants such as that described by Manktelow and Zuker. Manktelow, R.T. and R.M. Zuker, *Muscle transplantation by fasicular territory.* Plastic and Reconstructive Surgery, 1984. **73**: p. 751-757.

[6] A statistically significant delay in reinnervation was also seen between the end-to-side group with epineural window and that without.

fibres of a single motor unit will be of the same fibre type as the enzymatic and dynamic properties of a muscle fibre are dependent on the neuron innervating it [98]. Following withdrawal of neuronal input to a muscle (for example after traumatic division of its motor nerve) the individual muscle fibres undergo atrophic changes. Atrophy of the muscle fibres is marked - up to 35% loss of fibre diameter seen at only 2 weeks following denervation [93]. Atrophy occurs at the same rate and to the same degree irrespective of fibre type, and that following denervation the ability to distinguish between fibre types is lost [99][7].

During the process of reinnervation a single axon will form a new motor end plate with the first denervated muscle fibre that it comes into contact with. Furthermore any surrounding muscle fibres that are not innervated will induce collateral sprouting from the axon, resulting in a contiguous group of muscle fibres all innervated by the same axon and therefore part of the same motor unit (all of the same fibre type). This manifests itself as the classical fibre type grouping associated with reinnervated muscle [97] [98]. Experimental work has demonstrated that "slow" axons (associated with type I muscle fibres) regenerate more rapidly than "fast" axons (associated with type II muscle fibres) [8]

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[7] It has been argued however that fibre type distinction post denervation is retained, despite fibre atrophy, and that loss of distinct fibre typing is a feature of polyneuronal reinnervation of a muscle fibre. Mira, J.-C., *Effects of repeated denervation on muscle reinnrvation.* Clinics in Plastic Surgery, 1984. **11**(1): p. 31-38.

[8] As a consequence of this type II fibres may be reinnervated by slow axons, causing a reassignment of fibre type. This change in innervation, it has been suggested, leads to reversion of type II a or b fibres to a more primitive form, the type IIc fibre, which is histologically indistinct and it is this, not denervation, that accounts for the loss of fibre typing. Following re-growth of the fast axons (assuming intact basal laminae for directional axonal growth) the muscle fibres receive polyneuronal innervation (by both fast and slow axons), the end result being the recapture of the muscle fibres by the original fast axons and conversion from type IIc to their original fibre type designation (IIa or IIb). These experimental findings are not however in keeping with results of others that demonstrate loss of fibre type distribution in circumstances when reinnervation has not yet been achieved. Frey, M., et al., *Time course of alterations in muscle transfers with microneurovascular anastomoses.* Journal of reconstructive microsurgery, 1985. **2**(1): p. 33-43. Romanul, F.C.A., *Enzymatic changes in denervated muscle I. Histochemical studies.* Arch Neurol, 1965. **13**: p. 263-273..

and that as a result of this muscle fibre type characteristics and composition can be altered [93] [98]. With the restitution of neuronal input fibre atrophy is rapidly reversed with recovery of fibre diameter to or near pre-denervation levels[9] [98] [93].

1.3.5 Denervation and reinnervation changes – Physiological

Isometric twitch contraction displays both prolonged contraction and relaxation phases during the early stages of reinnervation, though this prolonged twitch response disappears with maturation of reinnervation. The time required for the development of maximal tension with repetitive stimulation is inversely proportional to stimulation frequency in normal muscle. In reinnervating muscle the development of tetany occurs at much lower frequencies, this being a function of more complete twitch fusion due to the prolonged relaxation phase. Transmission fatigue is the rate of decline of isometric tension developed by a muscle. In normal as well as reinnervated muscle this is proportional to the stimulation frequency, however in the case of the latter fatigue is observed to occur more quickly and at lower frequencies [100].

The recovery of function in reinnervated muscle is seldom to premorbid levels [101]. Experimental investigations have revealed several possible reasons why, despite restitution of neuronal input, reinnervated muscle fails to achieve its original physiological profile; Motor end plates in reinnervated muscle demonstrate morphological differences from those of controls, such units appearing much more primitive, even at 7 months post procedure [102]. The metabolic profile of muscle changes (such changes persisting for at least 10 months post operatively), with the energy metabolism demonstrating a reduced general activity and responsiveness to contraction similar to that seen in tenotomised or deconditioned muscle [103]. Satellite cell susceptibility to apoptosis would appear increased by denervation, resulting in impaired ability of muscle to reverse atrophic changes [104]. In addition to changes at a cellular level, residual populations of denervated fibres (that have not undergone

[9] It is worth noting that these findings only hold true for intact muscles subject to a crush injury to the motor nerve (axonotmesis rather than neurotmetsis).

atrophy) have been shown to be responsible for (weight adjusted) force discrepancies between reinnervated and control muscles [105].

1.3.6 Cross reinnervation

The histological typing of muscle fibres into types I, IIa and IIb corresponds with the physiological properties of the fibre. Type I describes slow oxidative (SO), fatigue resistant fibres typically found in the postural skeletal muscles (for example soleus). Type IIa are fast oxidative glycolytic (FOG), fatigue resistant (FR) fibres and type IIb fast glycolytic (FG), fatigable fibres (FF). The tetanic force output of the fibres is I < IIa < IIb, with cross sectional area of the fibres increasing in a similar fashion. Experimental determination of power output from individual SO, FF and FR motor units have shown tight clustering within a group and demonstrated little over lap between groups [106].

As stated previously the (re)innervating motor neurone influences the regulation of mechanical and metabolic properties of skeletal muscle [107] [108] [109] [110] [111] [112]. Transposition of the motor nerve supply from "slow" muscles to "fast" muscles has shown that it is possible to bring about a change in the fibre type composition of the affected muscles so that "fast" becomes "slow" and visa versa [113]. The phenomenon of fibre type grouping is seen in such reinnervated muscle, with islands of isotype fibres representing motor units supplied by a single motor neurone [106]. Following cross reinnervation the forces generated by the motor unit types tend to overlap and display a greater range of (and also increased) fatigability. In addition to the discrepancy seen between physiological properties of the motor unit and histological typing, the morphological properties of the fibres can also change, with significant increases in fibre size being noted experimentally between self reinnervated SO fibres and cross reinnervated SO fibres in the medial gastrocnemius muscle of the cat (MG) [106].

The degree to which a muscle assumes the fibre type characteristics of the nerve innervating is however not uniform across the published literature with some muscles demonstrating complete conversion of fibre typing, and others varying degrees of change [114] [113]. Interspecies differences are apparent from literature review on work undertaken in cats and rats, however inconsistencies in the subject muscles, and nerves

used to cross innervate them make comparison of results difficult [115] [116] [117]. Work undertaken by Buller et al. aimed to address these inconsistencies, and to determine whether individual muscles demonstrated varying degrees of resistance to fibre type reassignment. The results of their work indicated that the fibre type composition of the muscle influences the degree of fibre type change seen, as does the size and fibre type of nerve use to reinnervate it. Overall, it would seem, the extent of muscle speed (fibre type) transformation following cross innervation is dependent on the size of the cross reinnervating motor nerve (neurone population) in relation to the target muscle fibre number and assignment [118].

The transformation of muscle fibre type from one to another has implications clinically due to the differing twitch periods of the muscle fibre types [118]. For functional muscle transfers being used in the mimic system, spontaneous expression of emotion requires fast response muscle fibres [119]. Little work has been done on the cross reinnervating properties of the cranial nerves. It is known however, that there is quite wide diversity within the facial musculature in terms of fibre characteristics, and the facial nerve has the ability to induce muscle transformation following cross innervation [119] [120]. The possibility of functional outcome being influenced by the chosen branch of the facial nerve, as a result of differentiation of the transferred muscle fibres has been raised, though little data is available on the effects of cranial nerves on peripheral muscle [120] [119].

1.3.7 Muscle transfer

Muscle transfers can be orthotopic (being inset into their original tissue bed) or heterotopic (moved to a location other than its original tissue bed) grafts. Although only the latter type of transfer is analogous to the clinical situation, the former provides much information on how functional muscle transfers behave without having to contend with the "variables" of altered neuronal input, pattern of mechanical load and usage. Several free functional muscle transfer flap models have been used in experimental work, with most of these being undertaken in either the dog or the rabbit, a minority of studies using a primate model.

The first functional muscle transfer utilising microneurovascular anastomoses was undertaken by Tamai in 1970 placing transfers both orthotopically and heterotopically[10] [57]. This study demonstrated good histological results in terms of muscle structure at 5 months post transfer in all flaps that maintained vessel patency (70%). Those flaps in which vessels occluded post operatively suffered the same fate as that of the non-vascularised grafts described previously. Functional recovery of the transfers was assessed by EMG studies. These demonstrated that at 1 month post operatively a threshold stimulus of 2 – 4 times that of the control was required to evoke M-waves in the graft. It was noted that wave formation was nearly normal at 3 months, and by 5 months post transfer evoked potentials were similar to normal recordings, indicating "perfect recovery of function". No differences were noted between heterotopic and orthotopic transfers in terms of EMG or histology findings. This study was the foundation upon which clinical functional muscle transfers were based, with most authors reporting encouraging results [2] [1], as found by Tamai and colleagues.

In 1978 Terzis *et al.* examined how the individual steps undertaken in a muscle transfer (such as tenotomy and repair, microneurovascular repair, and orthotopic and heterotopic placement) affected outcome [75]. The results of this study produced several unexpected outcomes when compared to the clinical experiences being gained by those working in the field. Tenotomy and repair of the muscle reduced tetanic force at 14 months post operatively by 50%, though transfer of the muscle (either orthotopically or heterotopically) reduced force by 75%. The author postulated that microneurovascular repair (which reduced tetanic force by approximately 20%) and tenotomy produced a combined deficit effect resulting in the poor performance of the grafts. The conclusion was that useful recovery of muscle transfers was far less than had been anticipated. There were however several discrepancies in the study design (small numbers and minimal statistical examination), which may explain the difference between the results seen in this experiment and clinical observations.

Heterotopic muscle transfer of the rectus femoris muscle in the rabbit thigh (an identical model to that used by Terzis) was undertaken by Frey *et al.* examining both histological

[10] The model for this study was the gracilis muscle in the dog.

and functional aspects of outcome [78]. The findings of this study were markedly different from those of Terzis, with force production of 67.8% of the contra lateral control muscle when adjusted for weight at 6 months post transplantation. The study also demonstrated excellent correlation between intact fibre architecture and fibre type differentiation and force production. In another series, orthotopic transplantation of the same muscle produced a 32% recovery of tetanic function compared to the contra lateral control muscle at 90 days post operatively [58]. This demonstrated functional recovery over and above that of the grafts of Terzis [75] at approximately one fifth the recovery period, and half of the functional recovery of those in Frey's series [78] (at half the recovery period), giving weight to the previous findings of Frey [61] [80] [79].

Evaluation of muscle function following transfer to the face to reconstruct mimic function revealed that it was possible for branches of the facial nerve to power muscles larger in size than their original target organ [121]. Here it was possible to replace the scutuloauricularis muscle (the ear erector muscle in the rabbit) with a portion of the rectus femoris double its size, and produce a tetanic force of approximately 185% (107% weight adjusted) of the contra lateral control scutuloauricularis muscle. This phenomenon was also reproducible using the whole pectoralis decendens muscle (again about double the size of the scutuloauricularis), to produce 188% (131%) function of the contralateral muscle. The orthotopically transferred scutuloauricularis muscle regained 88% (84%) function in terms of tetanic force. It is of interest to note that whilst only 68% of function was recovered in a previous muscle transfer experiment in the periphery [78], a smaller muscle (traditionally thought to fare badly in functional muscle transfer procedures due to insufficient fibre numbers surviving the transfer [20]) placed centrally recovered 88% of function. Whether this represents a function of differential reinnervation ability between peripheral and central nervous systems, differential muscle response to transfer, or the fact that in larger muscles reinnervation takes longer as the distal portions of the muscle will be further away from the neurovascular hilum (and so there will inevitably be increased fibre atrophy and loss in a larger muscle compared to a small one in the same animal), was not addressed. The heterotopically transferred muscles (the aforementioned double sized piece of rectus femoris and the pectoralis descendens) experienced muscle mass reductions of between 20-25% in each graft, though no comment was made on what the possible outcome might have been if pieces

of the peripheral musculature the same size as the scutuloauricularis muscle had been the subject of transfer.

Further work on the central placement of oversized rectus femoris muscle pieces using the scutuloauricularis recipient site produced similar functional recovery figures for the indigenous muscle and a double sized portion of the rectus femoris (96% and 180% respectively). However a triple sized piece of rectus femoris returned only 173% of function compared to the contralateral control scutuloauricularis which, when adjusted for weight equalled 61% contrasting to 90% recovery seen with the smaller piece of peripheral muscle [122]. Rational as to why a triple sized piece of rectus femoris muscle failed to produce similar or greater raw force than a double sized portion was limited to a general discussion on the limitations of motor unit size that a single axon could reinnervate, and the inference that force deficit was likely to be due to a residual population of denervated fibres. No histological evidence for this was proposed.

Transfer of whole rectus femoris muscles into the cheek (again in the rabbit) and coaptation to the buccal branch of the facial nerve produced a 10% recovery of maximal tetanic force when compared to the contralateral thigh muscle [123]. Force measurements for the contralateral cheek muscles supplied by the buccal branch were unavailable, however given the small fibre mass of these muscles it is reasonable to hypothesise that even a 10% recovery of force in the rectus femoris muscle represented a larger raw force than that generated by the cheek musculature.

1.3.8 Proprioception

Regulation of muscle force and length are achieved by means of specialised sensory receptors within the muscle fibres and tendon substance. Golgi tendon organs, formed by encapsulated endings of large myelinated axons (Ib afferent fibres) situated at musculo-tendinous junctions, provide information about muscle tension to the CNS. When stimulated they produce an inhibitory effect on α and γ motor neurons. Muscle spindles are scattered throughout the muscle, both in the belly and at the musculo-

tendinous junction [124]. Each spindle consists of a number of highly specialised muscle fibres contained within a collagenous capsule; these fibres are described as intrafusal fibres as opposed to the vast majority of muscle fibres which are extrafusal. Muscle spindles receive efferent innervation from α and γ motor neurones and act via Ia and II afferent fibres. The systems acts so as to regulate muscle length and contraction [125].

Together with joint afferents and cutaneous receptors, the golgi tendon organs and muscle spindles provide information on both muscle and joint movement. There has been debate over which receptor(s) is the dominant sensory afferent in kinaesthesia. Most work done has looked at distal limb joints, particularly those of the hand, and although there is undoubted evidence for the role of joint and cutaneous receptors in kinaesthesia, marked deterioration in the ability to detect joint position occurs following the exclusion of muscle afferents [126].

Mimetic musculature is different from that of peripheral skeletal muscle. Typical fibre size is approximately 50% smaller than that of limb muscle, and type IIb fibres are rare in the facial muscles [127][11]. In addition, the proprioceptive mechanisms within this group of muscles appear different to those of peripheral muscles. Sensory apparatus with in the muscle fibres appear more basic (simple nerve end loops and knots) or have an atypical appearance [128]. Although typical muscle spindles have been demonstrated (both in human and animal preparations) these are fewer than in normal musculature [129] [128]. It is thought that the afferent fibres are trigeminal (V) in origin (though the possibility of VII fibres running via the V nerve cannot be discluded), and that most proprioceptive fibres from the facial muscles are conducted via the trigeminal branches, bypassing the trunk of the facial nerve. It is not known whether the γ motor neurone fibres (motor fibre for the intrafusal muscle fibres of the facial muscle spindles) travel over the facial or trigeminal nerve [130].

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[11] In Stennert's report on the morphology of human mimic musculature not only is a decrease in type IIb fibres noted, but the presence of type IIc fibres (usually associated with pathology) is also observed. It is postulated that this finding may reflect a difference in maturation between facial and limb musculature, or that enzyme investigation techniques may require modification when examining facial musculature. Stennert, E., et al. *Morphology of Human Mimic Musculature.* in *Proceedings of the Fifth International Symposium on the Facal Nerve.* 1984. Bordeaux: Masson Publishing.

The normal function of muscles is substantially dependent on their sensory apparatus [101]. Although as stated previously perception of muscle status relies not only on the intra-muscular sensory apparatus, but also on cutaneous and joint receptor cues, deficient reinnervation of muscle receptors and intrafusual fibres will affect the return of muscle function due to disruption of proprioception and reflex arcs [101]. Following denervation as part of a functional muscle transfer capsular thickening of muscles spindles has been noted, together with progressive atrophy of the intrafusal fibres [78] [131][12]. Following division and repair of the motor nerve to the cat soleus muscle muscle spindle numbers fall by 75%, together with a 55% decline in golgi tendon organ afferents. The use of a nerve graft results in almost 90% reduction of the sensory organs within the target muscle [132]. This atrophy and reduction in numbers of intramuscular sensory organs is in keeping with observations on muscle reflex behaviour following denervation and reinnervation with a neuromuscular pedicle. Findings in the sternothyroid (skeletal) muscles of rabbits, innervated by peripheral motor nerves, demonstrated absence of the myotatic reflex following successful reinnervation with a neuromuscular pedicle from the contra lateral, paired muscle, this is in keeping with previously published work showing that the myotatic reflex in absent in any muscle that is deafferented [133] [134]. In addition to loss of the reflex arc, loss of proprioception also results in deficient muscle force calibration in motor activities and causes problems with the acquisition of new motor skills [126].

[12] It is interesting to note that in rabbit rectus femoris transplants which underwent tenotomy and repair, together with microvascular repair, but had their motor nerves left intact, no changes in muscle spindle appearance were reported. Guelinckx, P.J., B.M. Carlson, and J.A. Faulkner, *Morphologic Characteristics of Muscles Grafted in Rabbits with Neurovascular Repair.* Journal of reconstructive microsurgery, 1992. **8**(6): p. 481-489..

1.4 Hypothesis

1.4.1 Facial Reanimation

When undertaking functional muscle transfers to the face, the transferred muscle mass is at least double that of the indigenous muscle[13] [10]. It is therefore apparent that a much larger number of muscle fibres are being employed to recreate the lost function than were originally present. Although both the muscle and nerve grafts used by individual clinicians tend not to vary (ie; relatively the same size piece of muscle and same diameter nerve graft are used) the branch of the facial nerve used as the source of reinnervation does. This means that relative to the nerve graft and muscle flap, the number of reinnervating axons changes from case to case.

From the experimental evidence presented in Giovanoli's work on muscle grafts coapted to cranial nerves it is known that the capacity for increasing motor unit size is less in cranial nerves than it is in peripheral nerves [122]. The above facts mean that if the transferred muscle were to attain full reinnervation of its muscle fibres then it would develop a force much greater than that of the small muscle mass it was attempting to recreate (and also a much larger force than that of the intact hemi facial musculature). However due to the decreased ability of cranial nerves to expand their motor unit size, the likelihood of the transferred muscle being anywhere near fully reinnervated is zero. The optimum result in facial reanimation is achieved when sufficient transferred muscle fibres are reinnervated that they balance the force production (and hence muscle excursion) of the contralateral side. With too few fibres reinnervated insufficient movement is the result. When too many muscle fibres are reinnervated within the graft not only is force production of the transferred muscle too great compared to that of the smaller muscle mass on the normal side (producing imbalance), but due to the lack of proprioceptive control spacticity may also develop. This is witnessed as the phenomenon of tightening described by many authors [40] [135] [16]. It is thought that in those transfers with little or symmetrical movement the balanced antagonistic action of the contra lateral facial musculature prevents such spacticity. This theory would accommodate not only the spectrum of results that are seen with facial reanimation

[13] This is the average weight of pectoralis minor versus the entire muscle mass of the hemi face, the muscle mass of those muscles involved in the action of smiling is a fraction of this figure.

surgery, but would also account for tightening, as initial symmetrical movement gives way to imbalance as increased neural ingrowth into the muscle recruits more muscle fibres causing a force production imbalance. In essence the result is determined by the size of the branch of the VII nerve used to reinnervate the muscle, or more specifically the number of axons reaching the muscle at the end of the cross facial nerve graft. Increasing numbers of axons produces better results up to a point where force production of the transferred muscle equals that of the contra lateral musculature. Beyond this a deleterious effect is seen with over activity and spacticity noted on the operated side. This has been seen clinically, and cases where flap reinnervation has been undertaken using the whole ipsilateral facial nerve stump have reported the highest rates of tightening [18] [40].

1.4.1 Peripheral reconstruction

In the periphery force production in functional muscle transfers have been noted to be disappointing compared to the contralateral musculature. The best results describe a maximum of 50% force recovery in the reconstructed site [43]. Whilst often due to anatomical constraints (parallel fibred muscles are used to replace pennate ones with inevitable loss of force production), the muscle mass transferred should usually be sufficient to accommodate for differential force production between the differing types of muscle anatomy. This is seldom clinically the case however. It has been postulated that incomplete reinnervation of the muscle may be responsible for this finding, possibly due to an insufficient reinnervating axonal load (ie; the recipient nerve is simply too small to fully reinnervate the muscle transfer) or that neuronal escape at the neurorrhaphy site robs the muscle transfer of motor axons. Improving the odds of muscle fibre reinnervation by increasing the axonal input should therefore result in fewer denervated muscle fibres and higher force production if the theory of residual denervated muscle fibres is correct.

1.5 Aims

This project aims to assess the effects of variation of axonal load on a constant sized vascularised functional muscle transfer in both the peripheral and central nervous system setting, to recreate the clinical scenarios of peripheral limb reconstruction and facial reanimation surgery. Specifically it wishes to address the effects that variation of axonal load has on outcome in the 4 following areas:

1. Physiology – To determine how an increased axonal load affects outcome (measured by maximal tetanic contractural force) when a large and small axonal load are compared in a cranial nerve setting[14]. To similarly assess the effect on outcome when the indigenous nerve and a double sized nerve is used in the peripheral nervous system.

2. Nerve Histomorphometry – Examination of neural profile post neurorrhaphy in terms of axonal number and total axonal cross sectional area to determine if increased axonal loads pre-neurorrhaphy result in improved neural profiles post-neurorrhaphy (defined as increases in both axonal number and total axonal cross sectional area) and to compare such profiles with physiological outcomes.

3. Immunohistochemical Muscle Studies – Assessment of neural ingrowth into the muscle transfer, using immunohistochemical markers, aims to allow direct analysis of the impact of an increased axonal load on the flap (as opposed to distal nerve segment histomorphometry providing indirect assessment of this). Additionally, it was sought to validate the use of such markers by comparison of these results with the findings of the physiology and nerve histomorphometry.

4. Muscle Fibre Typing – An additional aim was to assess the differentiation and transformation of muscle fibre types (together with identification of any features suggestive of fibre type grouping) in the transfers both intra and inter group, comparing such results with those of control muscles: Demonstrating any

[14] In this experiment the large axonal load is approximately 4 times the axonal number and cross sectional area of the small axonal load.

potential difference in fibre size and type as a result of firstly axonal number, and secondly the source of reinnervaton (cranial vs. peripheral).

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Chapter 2

Materials and Methods

2.1 Muscle transfer model

An animal model was employed for investigation into the reinnervation characteristics of functional muscle transfers. Previous studies [1-6] have used the rectus femoris muscle in the New Zealand white rabbit as a functional muscle transfer model. Due to the background data available, its favourable anatomy (distinct tendonous origin and insertion, single neurovascular pedicle) and the fact that the donor site defect is non crippling, this muscle was chosen for the current experimental work.

The rectus femoris muscle was transplanted to 1 of 4 orthotopic or heterotopic sites to provide a model analogous to the clinical setting being investigated. These sites were:

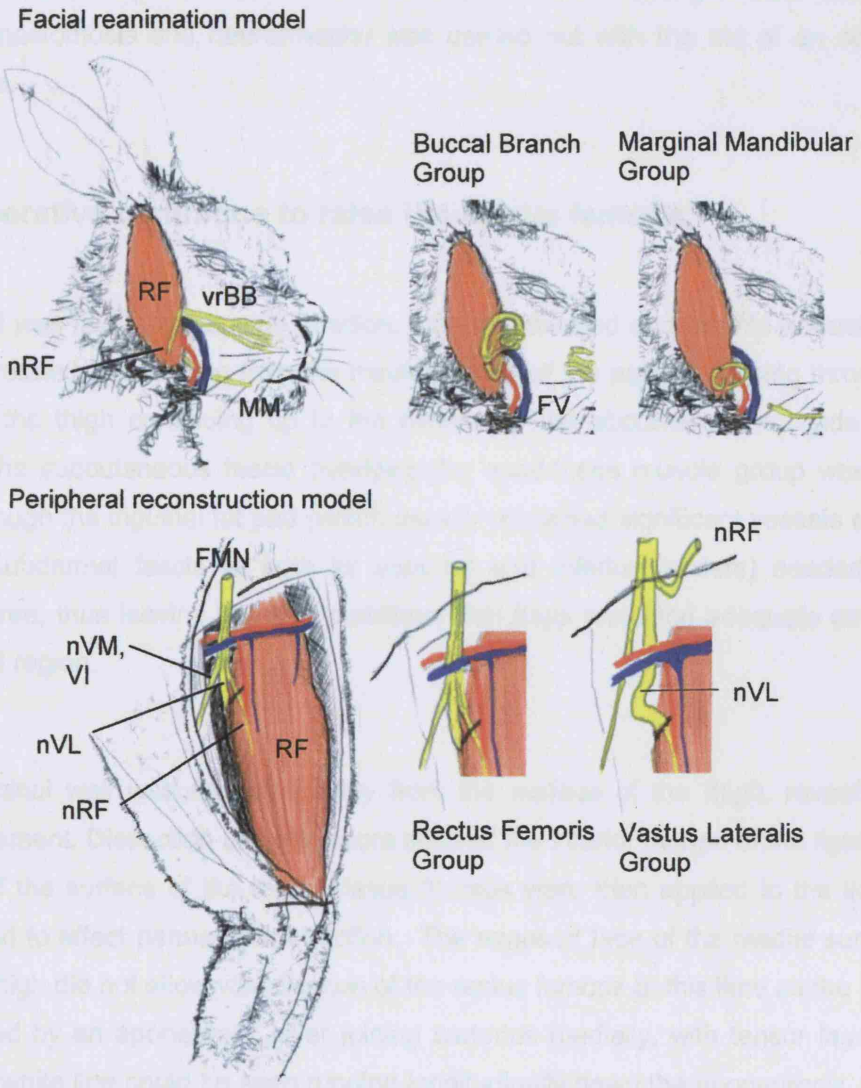
1. Orthotopic transfer to its original tissue bed with coaptation of the motor nerve to its original motor branch.
2. Heterotopic transfer to its original tissue bed, but with the motor nerve coapted to the larger motor nerve to vastus lateralis.
3. Heterotopic transfer to the right cheek pouch with coaptation of the motor nerve to the marginal mandibular branch of the facial nerve.
4. Heterotopic transfer to the right cheek pouch with coaptation of the motor nerve to the ventral ramus of the buccal branch of the facial nerve.

(The above is illustrated graphically in Figure 2.1.1)

In this manner, groups 1 and 2 examined how normal and larger than normal (double) reinnervating axonal loads affected outcome in the peripheral limb reconstruction setting. Groups 3 and 4 examined how differing axonal loads (small and large respectively) affected outcome in the setting of the central nervous system, analogous to procedures currently undertaken for facial reanimation.

Each of the 4 groups consisted of 12 animals. Half of the animals from each group were sacrificed, after physiological assessment as described below, at 6 months following muscle transplantation. The remainder were sacrificed at 9 months.

Figure 2.1.1: Diagrammatic representation of experimental muscle transfers. *(Upper)* Facial reanimation model, the rectus femoris muscle (RF) is transferred to the cheek with the vascular pedicle being the facial vessels (FV). The neural input to the flap is restored by coapting the nerve to the rectus femoris (nRF) to either the marginal mandibular branch (MM) or the ventral ramus of the buccal branch (vrBB) of the facial nerve. *(Lower)* Peripheral reconstruction model showing the muscle transfer reinnervated by either the original nerve or the neighbouring nerve to vastus lateralis (nVL). Also illustrated are the femoral motor nerve root (FMN), and the nerve to the vastus medialis and intermedialis muscles (nVM, VI).



2.1.1 Operative Technique

All operations were carried out in accordance with Home Office guidelines. Anaesthesia was accomplished with an intramuscular injection of 1ml Hypnorm (Jansen Animal Health, High Wycombe, Bucks, UK) as a premedication, following which 0.3ml intravenous diazepam (Phoenix Pharma Ltd., Gloucester, UK) was administered with intravenous Hypnorm given for maintenance as required. Each animal also received intravenous bolus of normal saline fluid totalling 30 – 50 ml over the course of the operation. Animals were shaved using electrical clippers to remove hair from the operative field pre-operatively. All neurovascular work including vessel dissection, vascular anastomosis and neurorrhaphy was carried out with the aid of an operating microscope.

2.1.2 Operative technique to raise the rectus femoris

The animal was laid in the supine position, left leg extended and the hip abducted and externally rotated. An incision from the medial border of the patella, running through the midline of the thigh continuing up to the midline of the abdomen, was made (figure 2.1.2.1). The subcutaneous fascia overlying the quadriceps muscle group was easily divided, though the inguinal fat pad (which usually contained significant vessels entering from the subdermal fascia at both its superior and inferior borders) needed to be dissected free, thus leaving two well mobilised skin flaps providing adequate access to the inguinal region.

The abdominal wall was retracted away from the surface of the thigh, revealing the inguinal ligament. Dissection using scissors allowed the inferior margin of the ligament to be lifted off the surface of the thigh. Tissue forceps were then applied to the ligament and secured to effect permanent retraction. The exposed face of the medial surface of the rabbit thigh did not allow visualisation of the rectus femoris at this time as the muscle was covered by an aponeurotic layer joining sartorius medially, with tensor fascia lata laterally. A white line could be seen running longitudinally down the aponeurosis, and by incising along this line from the medial surface of the patella three quarters of its length,

the underlying rectus femoris muscle could be exposed without peril to its pedicle. The rectus femoris was easily dissected out of its muscular pocket, and the muscle's distal insertion, into the patella tendon together with the rest of the quadriceps, was isolated.

The rest of the dissection was facilitated by the used of an operating microscope. The proximal end of the muscle, still covered by aponeurosis that is attached to the under surface of the reflected inguinal ligament, demonstrated a blue line (a vein) just under the surface of the fascial layer running horizontally across it. This vein drained directly into the femoral vein and acts as the venous pedicle for the flap, care must therefore be taken when approaching it. Using scissors the aponeurosis was opened cephalic to the vein, in a line running parallel to it. As a result of the traction on the inguinal ligament the aponeurosis and fibres of the muscles contributing to it parted easily to reveal a space in which the proximal end of the rectus femoris can be seen diving under the overlying psoas muscle. Blunt dissection in the plane between the two muscles, followed by retraction on the psoas muscle belly, reveals the origin of the rectus muscle: a superficial portion arising from the cranial iliac spine, and a deep portion arising from the ilium immediately cranial to the acetabulum [7]. Both heads of the muscle are bordered medially by vastus medailis, and laterally by vastus lateralis. Running proximally between rectus femoris and vastus lateralis muscles is the ascending branch of the lateral circumflex femoral artery and its accompanying vein – care must be taken when isolating the proximal tendonous insertion of the rectus femoris muscle not to wander too laterally and so divide this vessel.

Using micro scissors the remaining band of aponeurosis overlying the muscle was divided, then blunt dissection with micro forceps was used to develop a plane between the lateral circumflex femoral vessels running along the anterior surface of the rectus femoris, and the muscle fibres of tensor fascia lata laterally and sartorius medially. During this dissection it was necessary to diathermy small venules sprouting from the vein running into the aforementioned muscle fibres, and arterioles arising from the lateral circumflex artery, normally located deep and slightly cephalic to the vein. By this method it was possible to gain a good view of the anterior surfaces of the vessels. The medial boundary of dissection of the vessels was the femoral nerve (adjacent to the femoral

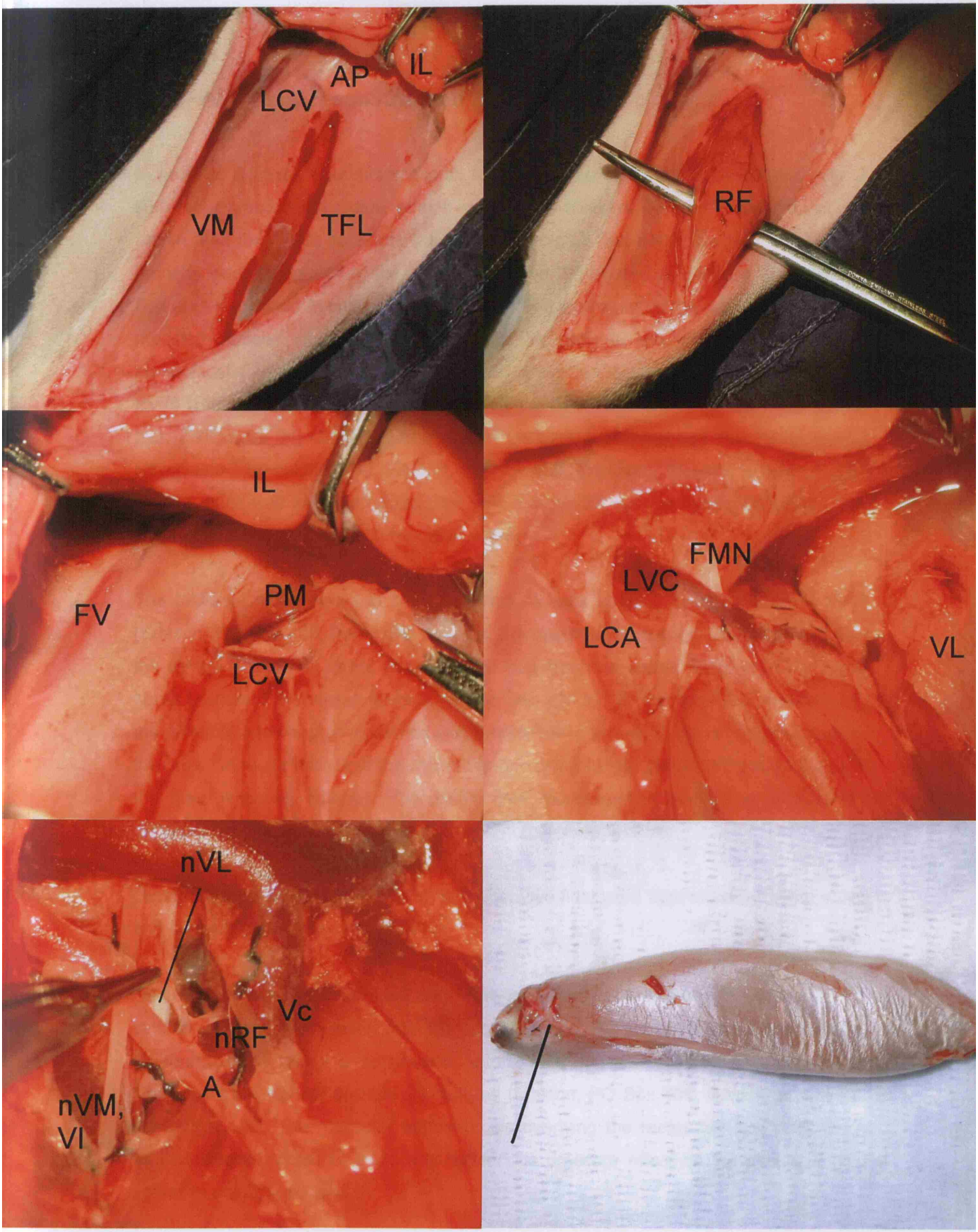
vein and artery), and the lateral border the medial surface of vastus lateralis (fused with the overlying tensor fascia lata).

The lateral circumflex femoral artery was safely tied off on the lateral border of the rectus femoris muscle (without deleterious effect on the thigh) prior to its bifurcation into the ascending and transverse branches. The vein was also ligated at this point, though care was taken not to compromise the drainage of the large vein found on the anterior surface of the muscle. The vessels were left intact and not divided until the flap pedicle had been defined, and the flap ready for elevation. One or two secondary pedicles entering the lateral surface of the rectus femoris (either from muscular branches to the vastus lateralis or direct from the ascending branch of the lateral circumflex vessels) were normally encountered. These vessels were uniformly minute and insufficient to sustain the muscle, and were easily dealt with by diathermy.

Working medially the motor branches of the femoral nerve were encountered (at the medial edge of the rectus) on the superior border of the vein, with the pedicle to the muscle located in the area caudal to its inferior border. Dissection of the pedicle was facilitated by gentle traction being applied to the rectus femoris so that it was laterally rotated, thus bringing the pedicle to a more anterior position. It is recommended that the lateral circumflex vessels be divided as close as possible to their origin on the respective femoral vessels, so as to provide the maximal pedicle length.

Prior to division of the origin and insertion the muscle was marked with a length of 3/0 braided silk (Ethicon, PO Box 408, Bankhead Avenue, Edinburgh, UK) so as to allow inset at the recipient site at the correct resting tension.

Figure 2.1.2.1: (overleaf) The dissection of the rectus femoris muscle. (Top,left): Exposure of the rectus femoris (RF) after incision of the muscular aponeurosis (AP) overlying it. The location of the lateral circumflex vessels (LCV), is indicated by a blue line (the vein) beneath the surface of the aponeurosis. The rectus femoris is bordered medially by the vastus medialis (VM) and laterally by the tensor fascia lata (TFL), which is fused with the underlying vastus lateralis (VL). The inguinal ligament (IL) is retracted with tissue forceps. (Top. Right): Dissection of the rectus femoris out of its pocket. (Middle, right): Following exposure of the lateral circumflex vessels, the aponeurotic layer is reflected laterally. The psoas muscle (PM) can be seen overlying the proximal insertion of the rectus femoris, and the femoral vessels (FV) are visible medially. (Middle, left): Division of the aponeurosis medially, together with division of sartorius fibres, reveals the femoral nerve motor nerve branches (FMN) above the lateral circumflex artery (LCA) and vein (LVC), and the femoral nerve at the medial border of the dissection. (Bottom, left): Isolation of the flap pedicle demonstrates the motor nerve branches to vastus medialis and intermedius (n.VM, VI), vastus lateralis (n.VL) and rectus femoris (n.RF). The nutrient artery (A) can be seen running to the muscle together with the medial vena comitantes (Vc). Forceps point to several communicating veins between the vena comitantes, and that accompanying the descending branch of the lateral circumflex artery. (Bottom, right): The vascular pedicle entering the medial surface of the muscle (line).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2.1.3 Peripheral reconstruction model

In order to evaluate how motor axonal load affects outcome in the clinical example of limb reconstruction the rectus femoris muscle was elevated as a free flap as described above, and then inset back into the bed from which it had been removed. The muscle tendons were reattached to their insertions using 2/0 braided silk sutures (Ethicon, PO Box 408, Bankhead Avenue, Edinburgh, UK), "figure of eight" at the proximal origin, and modified Kessler at the distal tendonous insertion to the patella. Microvascular anastomoses were performed to re-establish blood flow to the muscle using 10/0 nylon BV75 (Ethicon, PO Box 408, Bankhead Avenue, Edinburgh, UK) 7V43 (S&T Marketing Ltd., Zollstrasse 91, CH-8212, Neuhausen, Switzerland) interrupted sutures for arterial repair, and 11/0 nylon BV50 (Ethicon, PO Box 408, Bankhead Avenue, Edinburgh, UK) running back wall and interrupted front wall sutures for venous repair. Ischaemia time was not longer than 2 hours in any single case.

The (divided) motor nerve to the rectus femoris was coapted using 10/0 nylon BV75 (Ethicon, PO Box 408, Bankhead Avenue, Edinburgh, UK) interrupted sutures to either the (divided) proximal motor nerve branch supplying the rectus femoris, or the motor nerve branch to the vastus lateralis muscle was divided as far distally as possible and the proximal end coapted to divided distal motor nerve branch to the rectus femoris (with the proximal motor branch to the rectus femoris being turned proximally and sutured into the near-by psoas muscle). The above limb reconstruction techniques represented orthotopic and heterotopic models of muscle transfer respectively.

During the above procedure the flap and operative field were kept moist by saline soaks and direct irrigation with normal saline.

Once the muscle had been inset and microneurovascular anastomoses completed, the subdermal fascia was closed with 3/0 Vircyl (Ethicon, PO Box 408, Bankhead Avenue, Edinburgh, UK) using a continuous suture, and the skin closed with interrupted horizontal mattress 3/0 braided silk sutures (Ethicon, PO Box 408, Bankhead Avenue, Edinburgh, UK). The muscular apponeurosis overlying the rectus femoris in the thigh was not reconstructed to avoid any deleterious pressure effect on the muscle if post operative swelling occurred.

All animals received 0.3mls of subcutaneous Caprofen (), were wrapped in a blanket and observed until they had recovered sufficiently from anaesthesia to be placed back into their cage.

2.1.4 Facial reanimation model

In order to evaluate how reinnervating axonal load affects outcome in the clinical example of facial reanimation the rectus femoris muscle was elevated as a free flap as described above and transplanted to the cheek of the animal.

Once the muscle had been completely elevated to the point where it was solely attached by its neurovascular pedicle, it was covered with saline soaked gauze and the skin overlying the thigh closed over it using haemostats (so that it was now enclosed in a protective environment where traction on the vascular pedicle was precluded). Following this the recipient site for the flap was prepared.

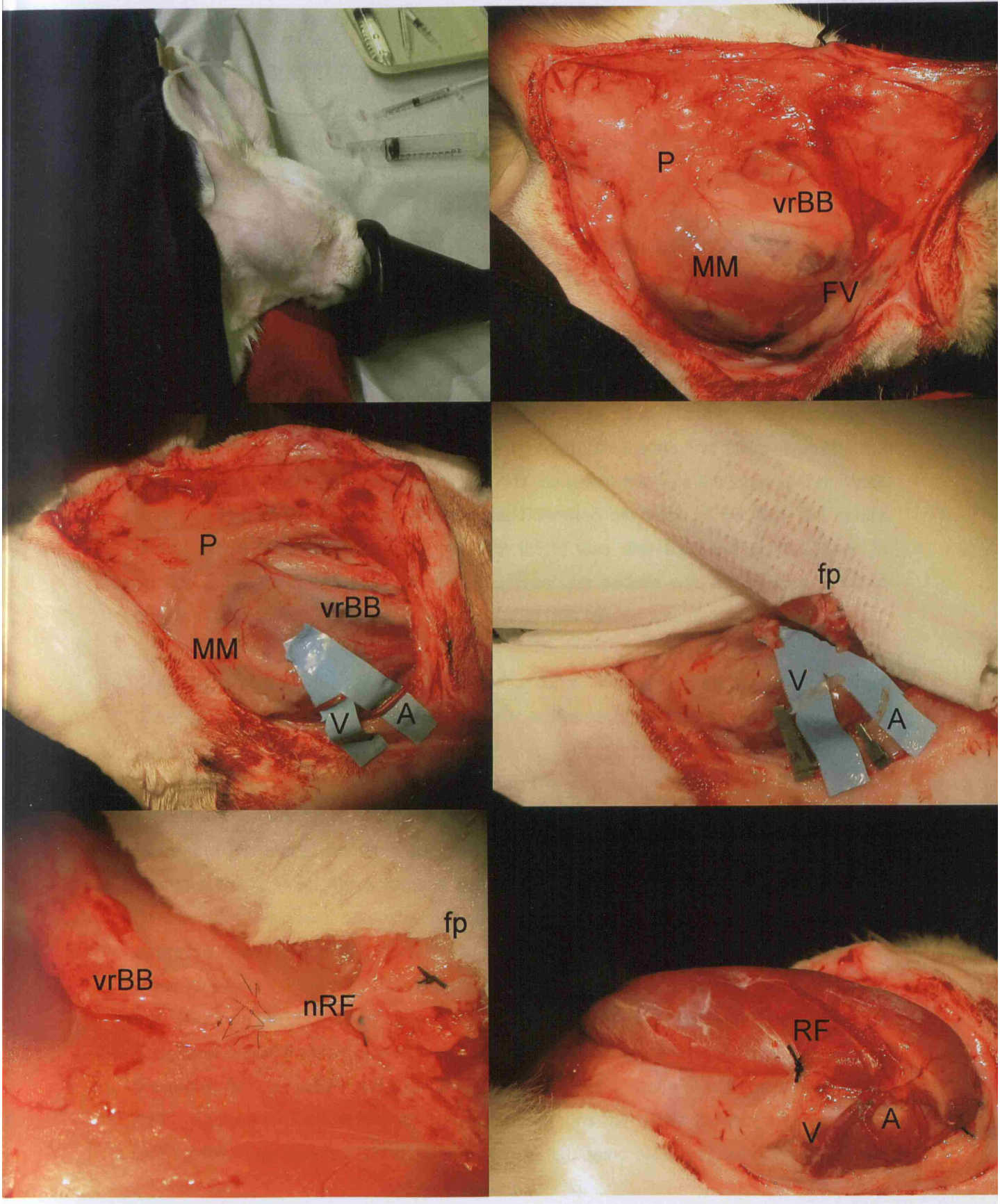
An incision through the skin running from the base of the ear to the mid-point of the inferior border of the mandible was made, and the resulting skin flaps elevated so as to provide good exposure. The facial vessels were identified, running up from the neck and hooking over the body of the mandible, and dissected out. The facial artery and vein were isolated for a length of 2 cms and prepared as recipient vessels.

To reinnervate the transplanted muscle either the marginal mandibular branch VII or the ventral ramus of the buccal branch VII was utilised. These were readily identified exiting the anterior border of the parotid gland of the animal in the fascial plane overlying the musculature of the face. Once identified the chosen branch was isolated and divided as distally as possible so as to provide as long a length pedicle as possible.

Microneurovascular techniques were applied as described above (section 2.1.3), though for technical reasons of accessibility neurorrhaphy was completed first, and venous anastomosis was undertaken using solely interrupted sutures (the same suture material was used as described previously). The flap was then inset using 2/0 braided silk

(Ethicon, PO Box 408, Bankhead Avenue, Edinburgh, UK) at the predetermined resting tension, the anchor points being the periosteum of the angle of the mandible, and the auricular cartilage. The skin was then closed using 3/0 braided silk (Ethicon, PO Box 408, Bankhead Avenue, Edinburgh, UK) horizontal mattress sutures. Postoperative care was as described above.

Figure 2.1.4: (overleaf) Transfer of rectus femoris muscle to cheek of rabbit. (Top left): The positioning of the animal for preparation of the recipient site. An incision made from inferior border of ear to midpoint inferior border mandible and skin flaps elevated to form the cheek pouch into which the flap is inset. (Top right): Identification of facial nerve branches – marginal mandibular branch (MM) and ventral ramus buccal branch (vrBB), the parotid gland (P) is visible at the root of the ear, and the positioning of the facial vessels (FV) is marked at the anterior border of the masseter. (Middle left): Identification and isolation of facial vessels – facial artery (A) and facial vein (V). (Middle right): Approximation of flap pedicle (fp) to recipient pedicle. (Bottom left): Neurorrhaphy – the ventral ramus of the buccal branch VII co-apted to the muscle motor nerve (nRF). (Bottom right): Microneurovascular anastomoses completed and flap inset – arterial anastomosis (A), venous anastomosis (V).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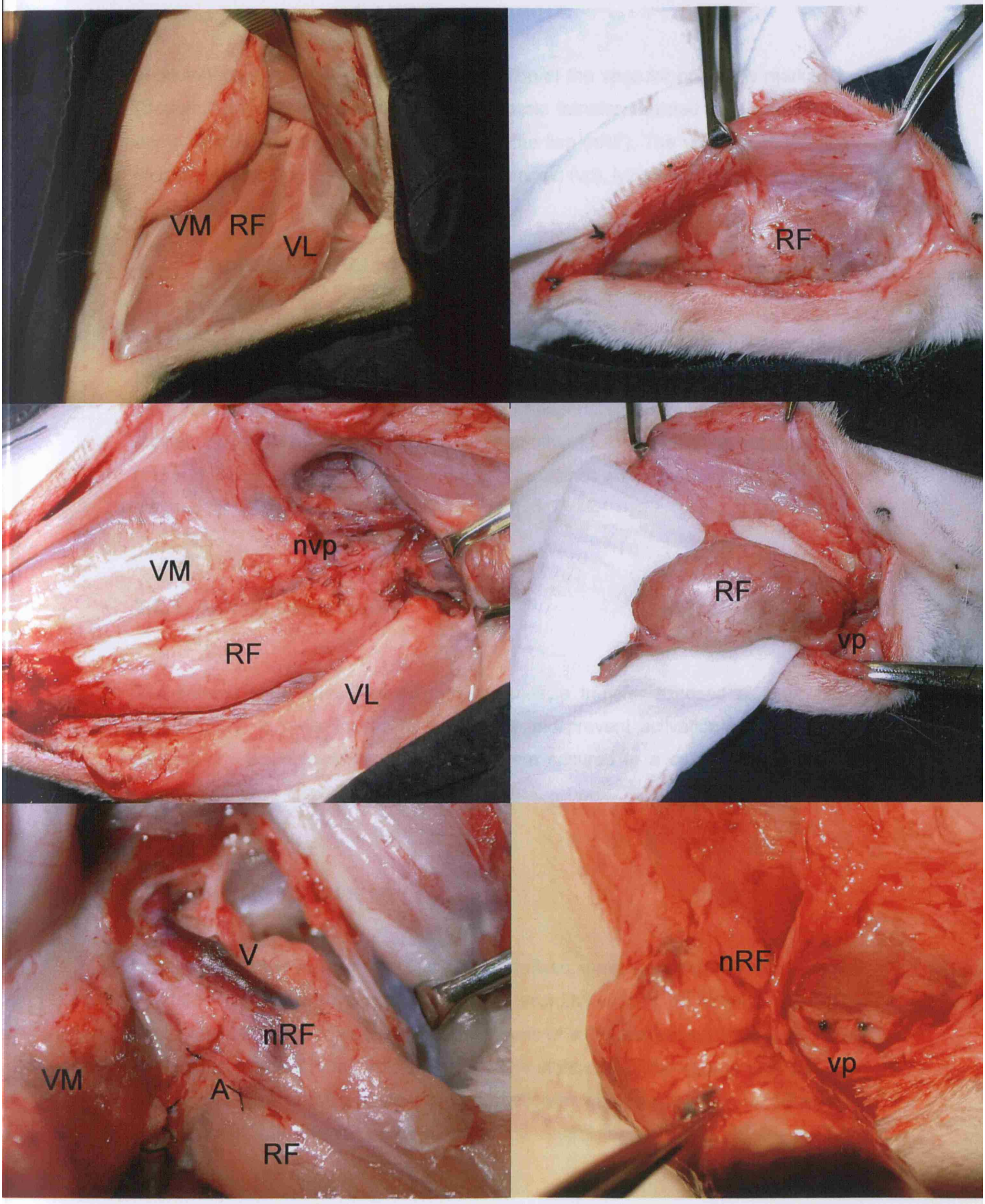
2.1.5 Flap harvest procedure

Flaps were harvested at 6 and 9 months post operatively for each of the four groups, half the animals being sacrificed at each time point.

Anaesthesia for the procedure was as described previously. Re-exposure of the transplanted muscle utilised the same incisions as described in section 2.1.2 for the peripheral reconstruction model and section 2.1.4 for the facial reanimation model. In elevation of the flap particular care was taken to identify the neurovascular pedicle as this region was often surrounded by scar tissue to a greater or lesser extent. Once the pedicle had been identified, the flap was dissected free of its tissue bed and isolation of the motor nerve branch undertaken. In the leg the distal insertion of the muscle into the patella tendon was divided, whilst in the cheek the whole flap was elevated so that it was only attached by its neurovascular pedicle. Following elevation of the flap, the contra lateral rectus femoris muscle (in the right thigh) was dissected out as described in section 2.1.2, though in this case the neurovascular pedicle was left intact and again only the distal insertion of the muscle into the patella tendon was divided.

Stimulation of the transplanted flap and the control rectus femoris muscle was then undertaken, with the animals subsequently euthanized prior to harvesting of tissue samples.

Figure 2.1.5: (overleaf) Harvesting of the transferred muscle. (Top left): Peripheral reconstruction model demonstrating moderate post operative scaring of the muscles vastus medialis (VM), retus femoris muscle transplant (RF) and vastus lateralis (VL). (Middle left): Peripheral reconstruction model demonstrating the sometimes severe post operative scarring involving both the muscle and the neurovascular pedicle (nvp). On close inspection, the motor nerve to the flap can be identified, having been dissected out. (Bottom left): Scarring demonstrated around the neurovascular pedicle of the peripheral reconstruction model. Heavy scarring around the artery (A), motor branch to the rectus femoris (nRF) and the vein makes isolation of these structures difficult. (Top right): Facial reanimation model - the rectus femoris flap (RF) in the cheek pocket 6 months following transfer. (Middle right): Muscle flap being dissected out of its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subcutaneous pocket in the cheek, the location of the vascular pedicle is marked (vp). (Bottom right): Facial reanimation model muscle transfer elevated prior to stimulation, demonstrating the nerve branch innervating the flap (nRF). The vascular pedicle is not visible in this photograph, but its position is marked (vp), lying directly under the flap.

2.2 Muscle Physiology

In order to assess the state of muscle function at 6 and 9 months following transfer physiological assessment of the muscle was undertaken. This form of assessment has been previously used to examine outcome in experimental functional muscle transfer studies [6] [8] [5] [3] and in studies looking at other factors influencing outcome [9] [10] [11] [12]. The stimulation protocol used by one author with experience in the field was adopted for use in the present experiment [13].

2.2.1 Animal preparation

The animals were anaesthetised and the muscle transfer exposed as described in section 2.1.1. In order to secure the animal and prevent activation of other muscles causing erroneous readings, the animals were secured to a custom made operating board, itself firmly attached to the operating desk.

2.2.2 Muscle stimulation

Once exposed and its neurovascular pedicle defined, each muscle transfer was attached to a force transducer coupled to a computer recording system. All muscles had a "free" end connected to the force transducer by means of a 2/0 braided silk (Ethicon) modified Kessler suture through the patella tendon. The other end of the muscle was the "static end", in the case of the peripheral reconstruction model the flap remained attached to the pelvis of the animal (held immobile by restraining straps), and in the facial

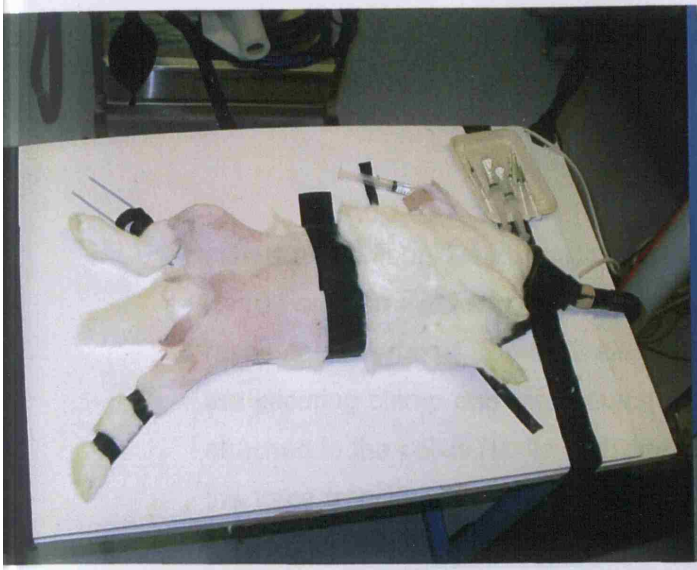
reanimation model the "static end" was connected to a fixed retort stand by means of a 2/0 braided silk modified Kessler suture (Ethicon).

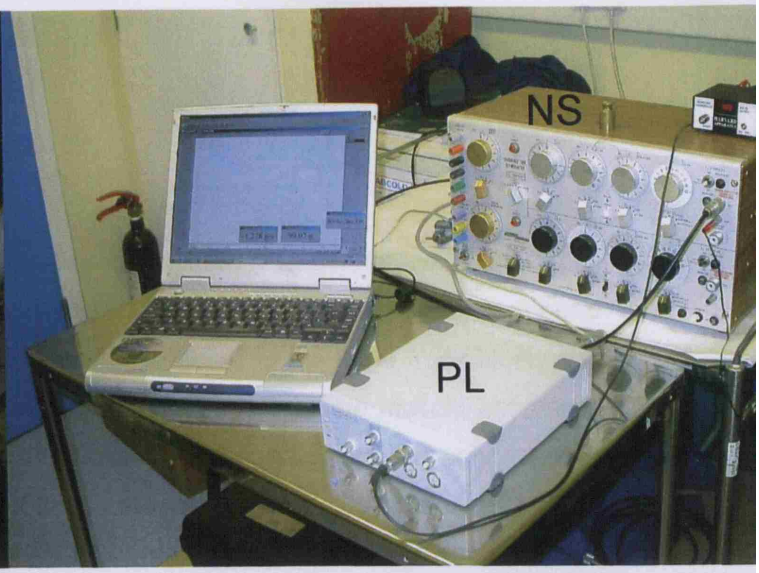
The motor nerve to the muscle flap, having already been isolated, was identified and the site of the neurorrhaphy located. Platinum stimulating electrodes (Grass Instrument Division, Astro-Med Inc., 600 East Greenwich Av., West Warwick, RI, USA) held 5mm apart in a custom made block were then applied to the surface of the nerve approximately 5 – 10mm proximal to the site of the neurorrhaphy. The stimulating electrodes were connected to a Grass S88 nerve stimulator (Grass, USA).

In order to record muscle contractions secondary to nerve stimulation the muscle was connected by the means described above to a Grass FT10 isometric force transducer held in a fixed position using a clamp and retort stand (Grass, USA). This was in turn connected to an ADI Instruments semi rectified bridge amplifier and an ADI Instruments Power Lab 2/20 (ADI Instruments,). This was connected via a USB cable to a laptop PC running Chart for Windows v4.2 software (ADI Instruments, UK), upon which data was recorded (Figure 2.3.1).

The stimulation protocol for the flap was based on previous authors work. Optimal resting tension for each muscle was assessed by measuring twitch contracture force over the range 100 – 170g tension on the muscle, with the tension producing the greatest twitch contracture being used as that under which the rest of the stimulation protocol was carried out. Electrical stimuli of 0.2ms duration were used, with 10V taken to be a suprathreshold stimulating voltage ([3] [4] [13]). Stimulation frequency of 100Hz was used to measure force generated in tetanus (muscle tetany typically occurs at 40 – 50Hz and therefore this represents suprathreshold stimulating frequency). All stimuli were applied for a period of 1 second with a 10 second gap between stimulations. During the period of stimulation the muscle was kept wet using normal saline solution warmed to 37°C. Details of the stimulation protocol used are given in Appendix 1.

Figure 2.2.2.1: (overleaf) Muscle stimulation. (Top left): Custom made operating board: note Velcro bands can be used to immobilise all 4 limbs, muzzle, neck, thorax and pelvis of animal. The board itself is secured to the operating table on which it rests also using Velcro straps. (Top right): The Grass S88 nerve stimulator (NS) used to

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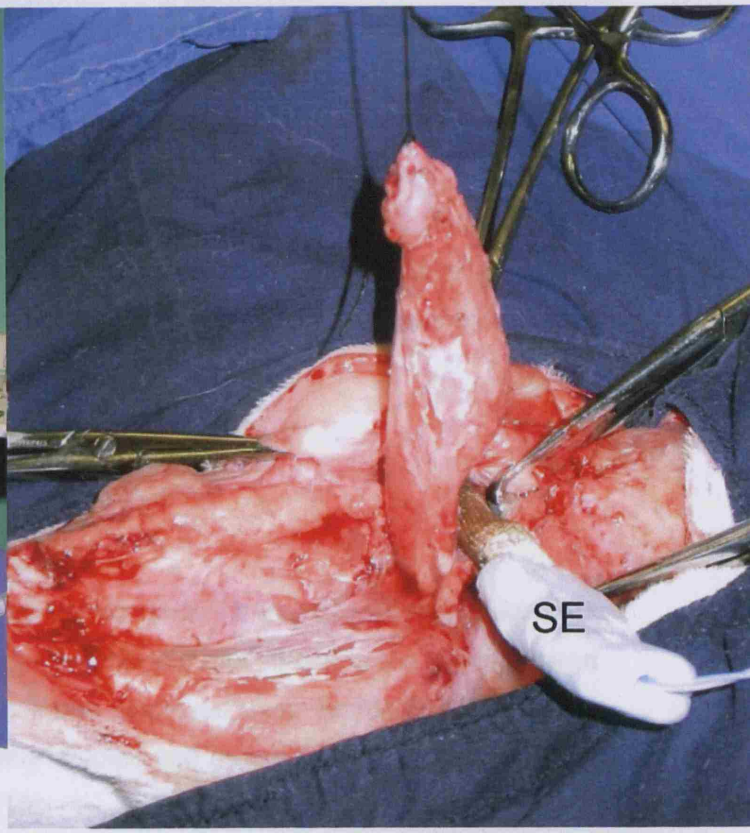
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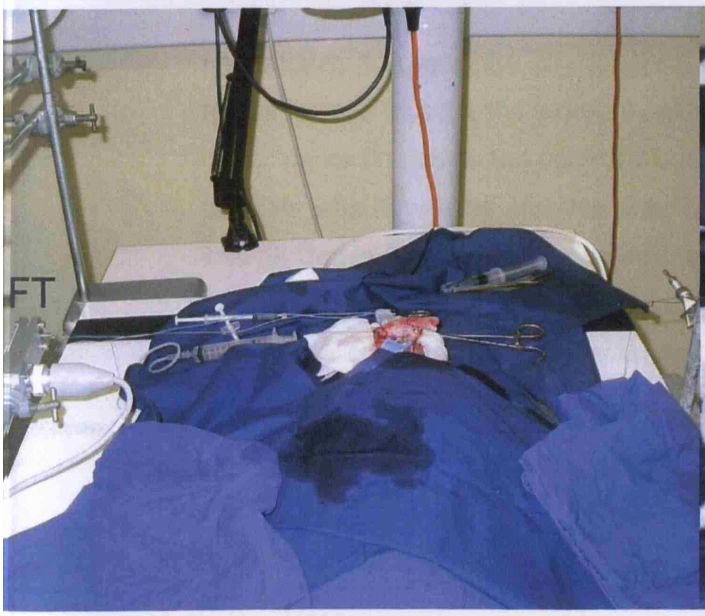
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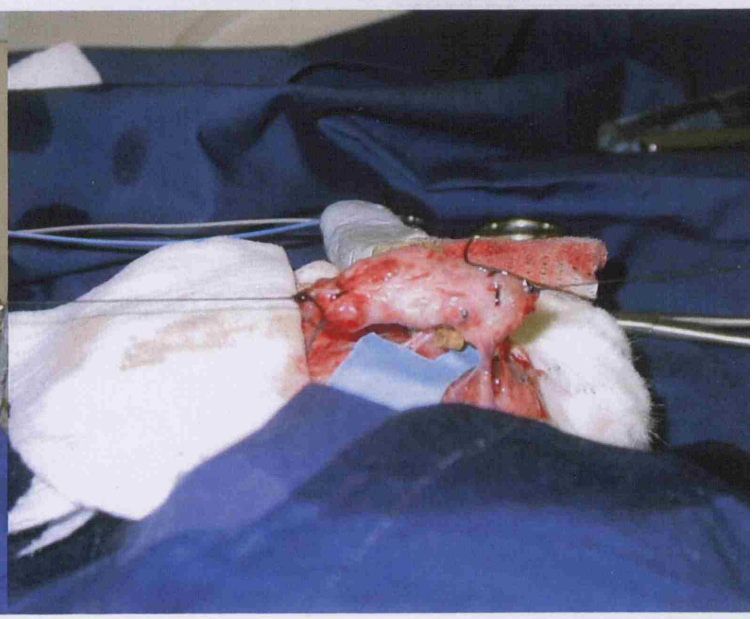


stimulate the flap. The Powerlab recording instrumentation (PL) connected to the Grass FT10 Force Transducer and a personal computer. (Middle left): Stimulation of peripheral reconstruction model demonstrating the muscle connected to the force transducer, and the securing clamp and retort stand mechanism. (Middle right): The muscle transfer is attached to the pelvis (static end) whilst the patella tendon (mobile end) is connected to the force transducer via means of a sturdy silk suture. The stimulating electrode block (SE) is placed under the motor nerve to the rectus femoris. (Bottom left): Stimulation of the facial reanimation model. The muscle is elevated free from the cheek pouch only connected by its neurovascular pedicle. The end of the muscle sutured to the angle of the mandible is secured, by means of a sturdy silk suture to a clamp and retort stand (static end). The end of the muscle sutured to the auricular cartilage is secured, by means of a sturdy silk suture, to the force transducer (FT) (mobile end). (Bottom right): Close up of the facial reanimation model during stimulation. The vascular pedicle is clearly visible in the foreground beneath the flap, with the stimulating electrodes placed under the motor nerve behind.

2.3 Nerve Histomorphometry

Assessment of biopsy samples from the motor nerves to the transplants was undertaken in order to determine total axonal number, diameter and total axonal cross sectional area both pre- and post- neurorrhaphy. Techniques employed were similar to the methods used in previous functional muscle transfer studies and others looking at nerve grafting and nerve morphology [4] [11] [12] [14] [15].

Immediately following the physiological assessment of the transferred flap and control rectus femoris muscle, the motor nerve branch to the flap was removed together with the motor nerve branch to the control muscle. As long a length of nerve as was physically possible was harvested and this was then divided into 2 sections. The operated nerve was divided in 2 at the neurorrhaphy site, and the proximal and distal segments labelled. These segments were then impregnated and embedded in epoxy resin as per routine protocol for semi-thin section preparation.

2.3.1 Nerve histology preparation

Immediately following harvesting of the relevant samples as described above, the nerve biopsy specimens were stretched out on a piece of labelled white card and placed in PIPES buffered 2.5% gluteraldehyde solution [16]. The sample was fixed in this solution overnight, following which it was washed in PIPES buffered 2% sucrose solution for 30 minutes and then placed in a 50:50 mixture of PIPES buffered 2% OsO_4 + 3% $K_3Fe(CN)_6.3H_20$ and PIPES buffered 6% $NaIO_3$ + 4% sucrose to osmicate the sample. It was left in this solution overnight. The sample was then sequentially dehydrated in alcohol and impregnated with epoxy resin following a previously published protocol [17] [18]. Following dehydration and impregnation with resin overnight, the samples were embedded in fresh epoxy resin, being placed in rubber moulds together with a label to identify them. These were then placed in an oven at 66°C with a vacuum pressure of 15psi applied overnight. The resin blocks containing the samples were then removed from the moulds and any excess trimmed off. The samples were then ready for sectioning. The details of nerve processing solutions and wash times are given in Appendix 2.

2.3.2 Nerve sectioning and staining

The resin blocks containing the nerve samples were sectioned using an ultra-tome (Riechert-Jung, Germany) and glass knives made in-house to produce 0.5µm sections. These were collected in a bath of 10% acetone (mounted on the glass knife used to cut the sections). The sections were then transferred to a clean glass slide upon which a drop of 10% acetone had been placed, and stretched using a chloroform soaked wooden stick. The slides were dried using a methanol lantern, following which stain and counter stain were applied [19].

Thionin stain was used as the myelin stain, with acridine orange used as the counter stain. A few drops of Thionin stain were placed over the samples and the slide gently heated over a methanol lantern for approximately 10s. The stain was then washed off with distilled water, and the acridine orange stain applied in a similar manner to the

thionin. This too was washed off with distilled water, and the slide dried on a hotplate. Once dry a cover slip was mounted over the sections using epoxy resin.

2.3.3 Nerve morphometry

Once the nerve sections had been mounted on slides and appropriately labelled digital images of them were captured at x40 magnification using a Zeiss Axioskop 20 microscope (Carl ZeissLtd., Welwyn Garden City, UK) with a Leica DC 200 digital camera (Leica Microsystems Ltd, Heerbrugg, Switzerland) mounted on it. Leica DC Twain image capture software (Leica, Switzerland) was used to record the images in greyscale at 1798 x 1438 pixel resolution. Sequential images were recorded so that the entire field was captured, and then the images were put together as a photomontage using Adobe Photoshop v6.0 (Adobe Systems Incorporated, USA). The image was then expanded to 250% of original size using Hewlett Packard Design Jet 500 PS (Post Script) rip printing software (Hewlett Packard, USA) and printed on 42inch roll paper using a Hewlett Packard Design Jet 500 PS large format printer so that final magnification of the image was x2250.

Axon counting, diameter and area assessment was undertaken using a semi-automated computer analysis system, consisting of a digitising tablet (GTCO, Columbia, MD, USA) coupled to a personal computer, with the data recorded in Microsoft Excel.

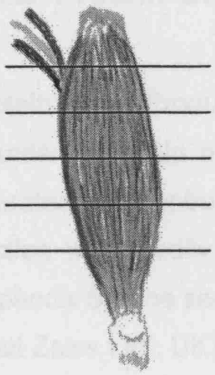
2.4 Muscle morphology

Immediately following animal sacrifice and motor nerve harvesting, both the operated and control muscles were removed from the carcass. Each muscle was weighed and its length measured. The muscle was then cut into 6 equal sections using a fresh skin graft knife (Figure 2.4.1), each section being numbered in a standard fashion from proximal to distal. These whole muscle sections were then mounted onto cork discs with the aid of gum tragocanth, and snap frozen in isopentane that had been frozen in liquid nitrogen and was now at the stage of melting. Each sample was left in the isopentane for approximately 90 seconds to ensure it was thoroughly frozen, following which it was

retrieved from the isopentane, wrapped in labelled aluminium foil and placed directly into liquid nitrogen for transport to its definitive storage site (a -80°C freezer in RAFT). The muscle was stored in this condition until it was able to be processed.

As soon as muscle was needed for processing, the relevant sample(s) were retrieved from the deep freeze and thawed in 10% buffered formalin solution (in-house preparation at RAFT). Once fixed in the solution for 48 hours, the samples were then processed using an automated machine and embedded in paraffin blocks for sectioning. The reason for initial frozen storage of tissue was because of a lack of information on the suitability of the immunohisotchemical reagents on paraffin impregnated rabbit tissue. Subsequent protocol development in house at RAFT determined that formalin fixation at the time of tissue harvest would have been equally suitable, though this was unknown at the initial stages of the project, and so it was deemed best that fresh frozen samples be taken to keep all tissue processing options open. The details of paraffin tissue processing and fixation are given in Appendix 3.

Figure 2.4.1: Muscle sections taken for histology. All muscle sections were numbered from proximal to distal (the pedicle easily identifying the proximal origin of the muscle). The face of the tissue block examined was always the proximal one. The identity of each muscle section is shown, with the histological examination detailed for the relevant sections.



2.4.1 Muscle Fibre Typing

Standardised mid muscle 10μm paraffin sections (muscle section 4) were mounted on Surgipath Snowcoat X-Tra glass microscope slides (Surgipath, St. Noets, Cambs., UK). Commercially available monoclonal antibodies were used each coupled to a differing visualisation system (ie; secondary antibody colour). By application of this method it is possible to distinguish type I (black), IIa (pink – grey) and IIb (pink) muscle fibres. Full details of the protocol for this procedure are given in Appendix 4.

The stained muscle sections were examined at X 200 magnification using a Zeiss Axiophot (Carl Zeiss Ltd., UK) light microscope coupled to a custom made digital image capture device and computer software (Medical Imaging Dept., Gray Cancer Institute, Northwood, Middlesex, UK), and analysed with custom modified Image J Freeware image analysis software (US Natioal Institute of Health, Bethesda, Maryland, USA). Four random fields were chosen from each section and the muscle fibres classified as either type I, IIa or IIb. The cross sectional area of each fibre was also measured. In this way approximately 275 muscle fibres were counted in each section. This method has been previously used by several authors, and taken to be representative of the muscle as a whole. Sections were examined in every operated muscle, with a panel of 6 randomly chosen control muscle sections used to provide normal values.

2.4.2 Protein Gene Product 9.5

Protein Gene Product 9.5 (PGP 9.5) is a neuron – specific cytoplasmic protein [20]. Standardised mid muscle sections (muscle section 4) were taken from each of the operated and control muscles and stained for PGP 9.5, so that neural tissue within the section was readily identifiable (the protocol for PGP 9.5 tissue staining is given in Appendix 5). The sections were examined at X 200 magnification using a Zeiss Axiophot (Carl Zeiss Ltd., UK) light microscope coupled to a custom made image capture device, and analysed with custom made image analysis software (Gray Cancer Institute, UK). The section was examined in a systematic fashion so that every field was inspected and the total number and size of the nerve bundles within the muscle section was recorded.

Due to the nature of immunohistochemically stained tissue, semi-quantitative analysis of the nerve bundles was performed and a modification of previously published methods for grading PGP 9.5 staining in muscle flaps was used [21] [22]. Each of the nerve bundles was assigned into one of 3 groups according to its maximal diameter (as not all bundles were perfectly round), these being those measuring less than 75µm at their maximal diameter (+), those between 75µm and 150µm (++), and those measuring over 150µm (+++) (Table 2.4.2).

Table 2.4.2: Grading of PGP 9.5 stained nerve bundles within muscle sections.

Nerve Bundle Diameter	Score
< 75 µm	+
75 - 150 µm	++
> 150 µm	+++

The number of bundles in each section was multiplied by their respective scores to give an overall score for the muscle section. Each operated muscle was normalised by the contra lateral rectus femoris muscle so that the total PGP score for each transplanted muscle was expressed as a percentage of its control. This percentage value was used as an index for reinnervation of the muscle transplant and allowed direct comparison of individual flaps as each was normalised by its own control (Figure 2.4.2.1)

Figure 2.4.2.1: Reinnervation index calculation. Ao denotes the number of nerve bundles identified < 75 µm in diameter (+) in the muscle transfer, Bo those 75 - 150 µm (++) and Co those > 150 µm (+++). Ac, Bc and Cc denote the same values as above seen the control muscle.

$$\text{Reinnervation index} = \left[\frac{(Ao \times 1) + (Bo \times 2) + (Co \times 3)}{(Ac \times 1) + (Bc \times 2) + (Cc \times 3)} \right] \times 100$$

2.4.3 Neural Cell Adhesion Molecule

Neural cell adhesion molecule NCAM is a cell surface glycoprotein that is found in abundance along the sarcolemma of embryologic, foetal and postnatal skeletal muscle. In mature skeletal muscle its expression is limited to the acetylcholine receptor at the neuromuscular junction, however following denervation it reappears within the cytoplasm of the cell [23]. Standardised mid – muscle sections (muscle sections 2 and 4) were taken from both control and operated muscles and stained for NCAM (the protocol for tissue staining with NCAM is given in Appendix 6). The sections were examined at X 200 magnification using a Zeiss Axiophot (Carl Zeiss Ltd., UK) light microscope coupled to a custom made digital image capture device, and analysed with custom made image analysis software (Gray Cancer Institute, UK). The whole section was examined and using semi automated, computed assisted analysis and the percentage of denervated (NCAM +ve) muscle fibres was scored in a similar fashion to that of the PGP 9.5 sections (Table 2.4.3).

Table 2.4.3 : Grading of muscle sections stained with NCAM for the percentage of denervated muscle fibres.

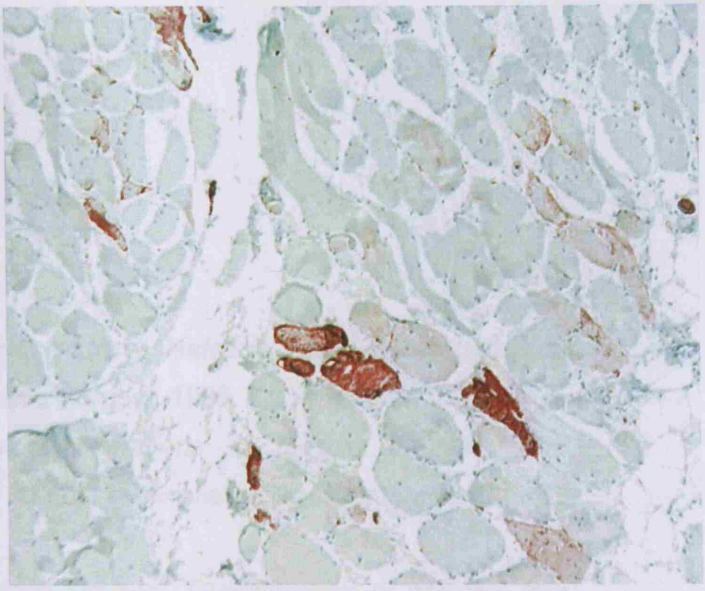
Percentage NCAM +ve fibres	Score
< 1%	1
< 10%	10
10 – 20%	20
20 – 40%	40
> 40%	50

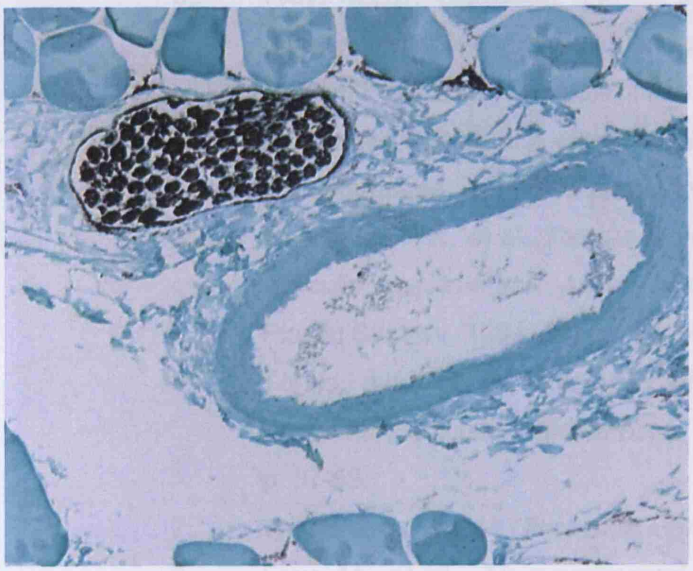
The mean score for each muscle was calculated from the 2 muscle sections examined to give a "denervation score" for the muscle. Both operated and control sections were studied, though expression of N-CAM was minimal (as would be expected) in the control muscles, being uniformly <1. Direct comparison of the mean values from the operated muscles was therefore undertaken.

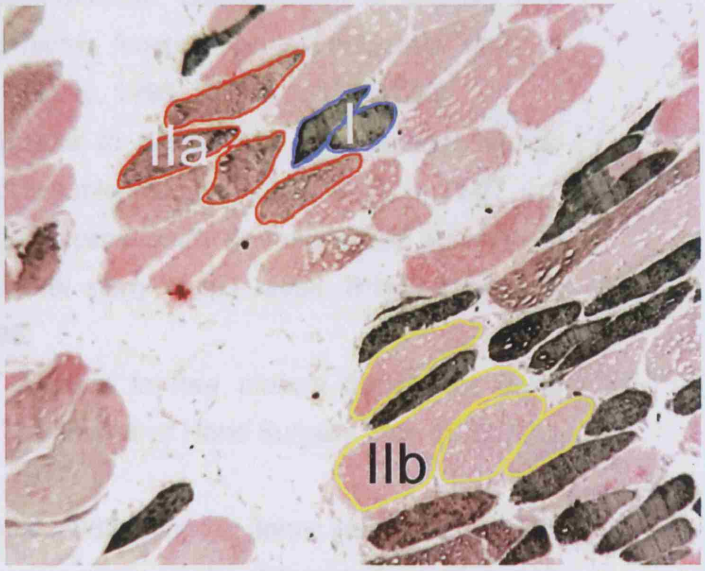
Figure 2.4.3.1: (overleaf) Tissue preparation sections and physiology assessment. (Top, left): Nerve section image (greyscale) recorded using a digital camera mounted on a microscope and image capture software. The myelinated axons are clearly visible, with the internal circumference of the myelin sheath being measured to provide axonal cross sectional area (demonstrated here as a yellow trace). In the centre of the image a blood vessel is seen. (Middle, left): Muscle section stained for PGP 9.5. A medium sized nerve bundle (++, [75 - 150 μm]) staining positive for PGP 9.5 is clearly visible demonstrating discrete axons. (Bottom): Muscle physiology measurement using Chart for Windows. Muscle contractions are seen as the blue trace, with force in grams on the y-axis of the graph and time on the x-axis. The 2 contractions on the far left are maximal tetanic contractions (100Hz), subsequent contractions are at stimulus frequencies sequentially increasing from 10Hz to 100Hz (increments are delineated by a dotted vertical line). (Top, right): Muscle section stained for NCAM, denervated myocytes stained as red. (Middle, right): Muscle fibre typing. Examples of fibre types are labelled together with an outline trace of same type neighbouring myocytes. Example type I fibres (staining black) are outlined in blue, type IIa (pink-grey) are outlined in red and type IIb (pink) are outlined in yellow).

2.5 Statistical analysis

Statistical analysis of data was undertaken using Sigma Stat Version 2.0 (Jandel Scientific Software, Jandel Corp., USA). For parametric data a non paired t-test was used, for non parametric data a Mann Whitney rank sum test was employed.



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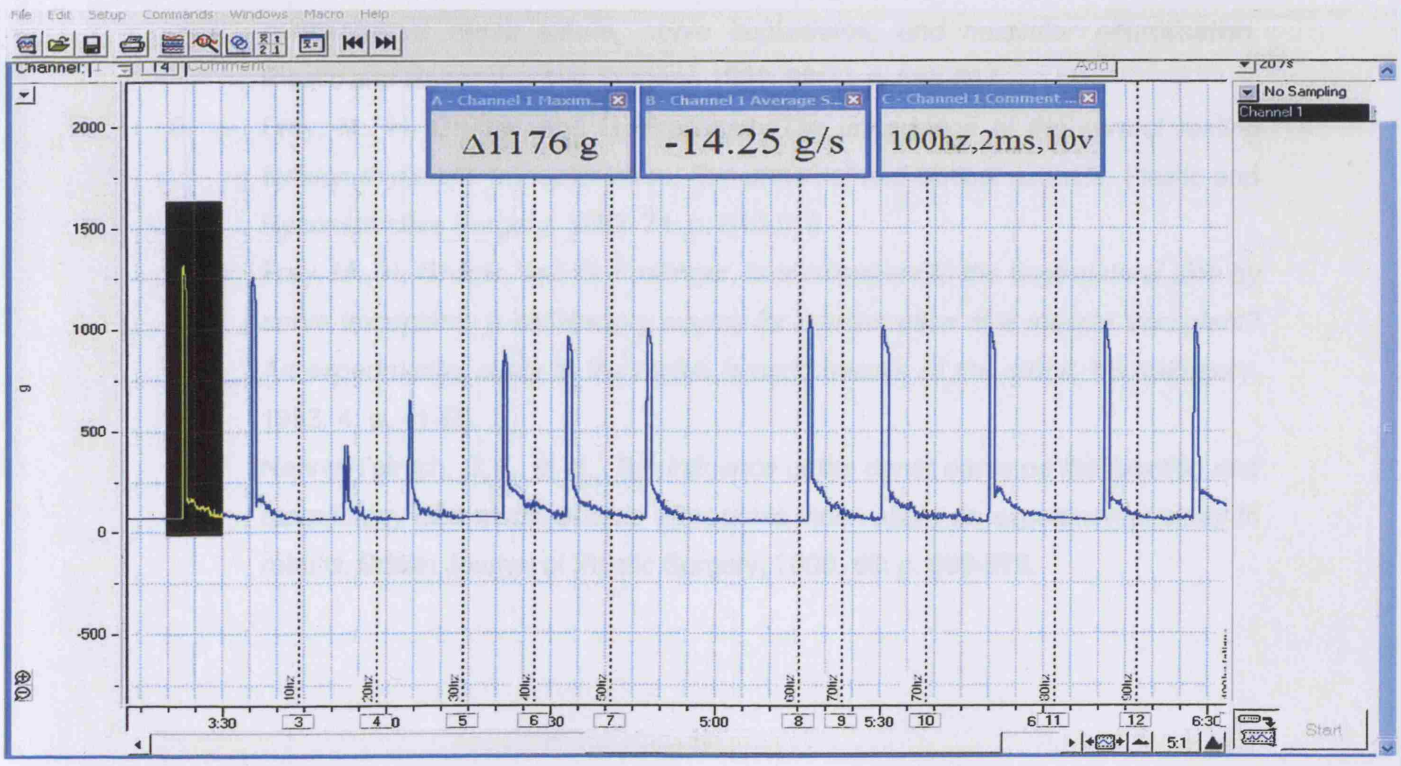
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Chapter 3

Results

Physiology

3.1 Introduction

Functional recovery following the transfer of muscle with the use of microneurovascular techniques has been the subject of investigation and debate since its first clinical implementation [1] [2]. Although clinical experience pointed to the potential of this new technique (as did the initial experimental findings [3]), preliminary investigations into force generated by transplanted muscle indicated that recovery of performance post transfer was so poor, these results called into question the viability of the technique [4]. The inconsistency between clinical and experimental observations lead other groups to examine how the process of denervation, ischaemia and tenotomy affected muscle, either as individual insults or in combination [5] [6] [7] [8]. These results provided greater encouragement to those interested in developing the use of neurovascular muscle flaps for reconstruction of damaged or absent muscles. Reassessment of the force produced by a muscle post transfer demonstrated that whilst full recovery of function was unattainable (when compared to the unoperated contralateral muscle) significant use remained in the organ (contradicting the previously established experimental findings) [9].

Functional muscle transfers are heterotopic when used in the clinical setting (ie: the muscle is not replaced into its original bed, but moved else where). A limitation of the aforementioned studies is that they examined the muscles after orthotopic placement (they had been placed back into their original location). As detailed previously in Chapter 1 the nerve to which a muscle is coapted following denervation plays an important part in determining how the graft will ultimately function and therefore a more clinically pertinent question is how muscle transplants function after they have been coapted to a foreign neural input. Relatively few studies have been published on the functional ability of muscles following transfer to a heterotopic site [10] [11] [12] [13] [14]. Although the limitations on the mass of muscle a given nerve can reinnervate have been explored [10], it is the reverse scenario that can be the clinical problem. Most facial reanimation techniques either use whole muscle (pectoralis minor) or a trimmed portion of muscle (gracilis or latissimus dorsi), with the size of the graft remaining fairly constant (the fascicular territory dissected out by individual surgeons does not tend vary hugely between cases, such that it is unlikely that it is the size of the transferred muscle which results in variability seen in outcome). Similarly in peripheral limb reconstruction whole

muscles tend to be transplanted with certain muscles being indicated for particular reconstructions, again the muscle mass being transferred remaining relatively constant between patients[1]. In such circumstances the biggest factor being varied (as, for the reasons given above, the muscle transfers tend to be of constant size within the practice of individual surgeons) is the motor nerve used to reinnervate the transplant. To date there has been little work examining how variance of axonal input affects a constant sized muscle transfer.

3.2 Aims

To compare the effect of differing axonal loads, one large and one small, on a standard vascularised muscle transfer using physiological studies as the outcome measure.

To evaluate how such loads influence outcome at 2 different time points (so as to determine whether late onset tightening in facial reanimation may be related to a variance in axonal load).

To compare and contrast the physiological outcomes seen when utilizing a cranial motor nerve and a peripheral motor nerve as the reinnervating source in the aforementioned settings.

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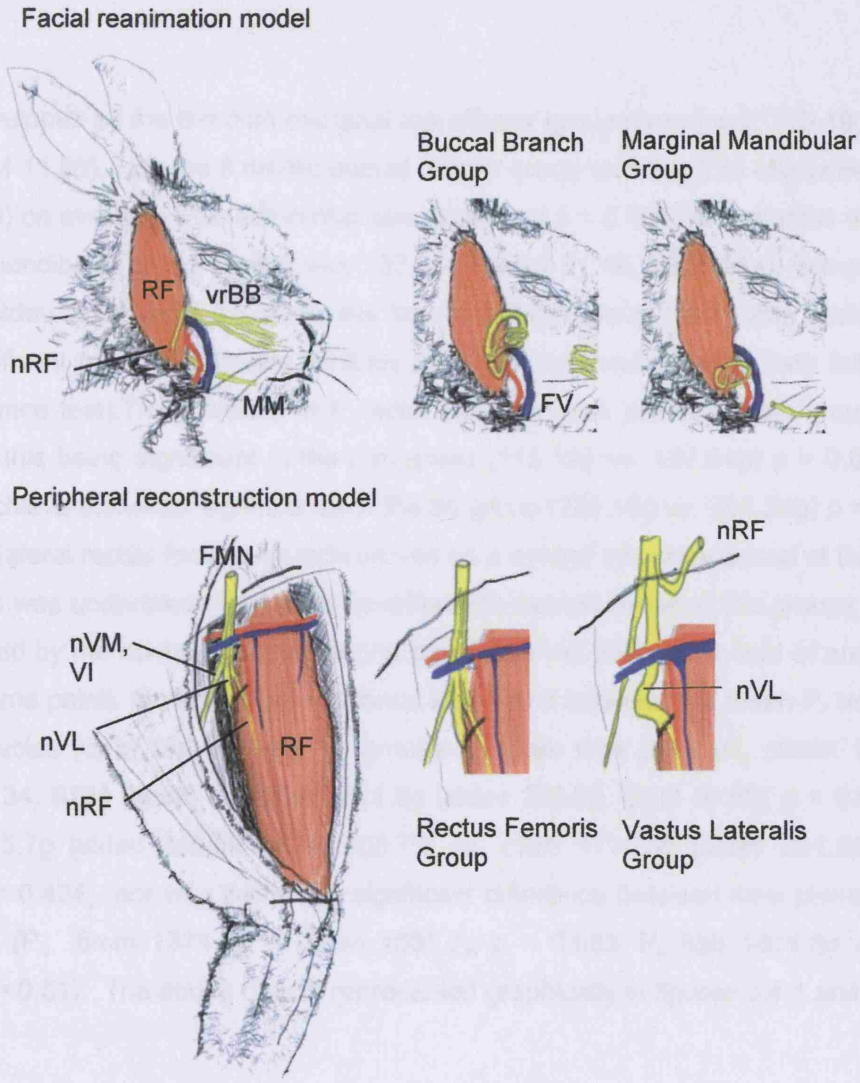
[1] This is based on the assumption that individuals within the population when matched for age and sex will display a reasonable degree of concordance in terms of muscle shape and mass for a given donor unit (ie: the ratios for the gracilis muscle will be fairly constant across the population when examined for mass compared to total body mass, and length compared to total body length) and that neuronal supply to the muscles is stable in a similar fashion.

3.3 Methods

Forty eight male, 3.0 – 3.5 kg New Zealand White rabbits were divided into 4 groups of 12 animals. The groups were designed to emulate the clinical scenarios of peripheral limb reconstruction and facial reanimation utilizing functional muscle transfers. The rectus femoris muscle was isolated on its neurovascular pedicle (as detailed in Chapter 2 section 2.1.1) and then transferred to one of 4 recipient sites. In the facial reanimation model the muscle was transplanted to the cheek pouch of the animal and vascular repair undertaken anastomosing the flap vessels to the facial artery and vein. The marginal mandibular branch (Group 1) or the ventral ramus of the buccal branch of the facial nerve (Group 2) were used to reinnervate the flap (Figure 3.3.1). In the peripheral limb reconstruction model the muscle was placed back to its original site and anastomosed to its anatomic vessels. The motor nerve to the muscle was then coapted to either that of the vastus lateralis (a heterotopic graft) or to its anatomic nerve (Figure 3.3.1). Half the animals from each group underwent physiological assessment at 6 months post operatively, the other half at 9 months post operatively.

At the time of physiological assessment the muscle transfer was carefully dissected out of its bed, and again isolated on its neurovascular pedicle (Chapter 2 section 2.1.4). The muscle then underwent physiological assessment as described in section Chapter 2 section 2.2.2.The data was recorded using a force transducer and a personal computer. Chart for Windows software acted a both a capture device and analysis tool for muscle stimulation readings. Statistical analysis of the data was undertaken using Sigma Stat software. The schedule for muscle stimulation is detailed in Appendix 1.

Figure 3.3.1: Diagrammatic representation of experimental muscle transfers. *(Upper)* Facial reanimation model, the rectus femoris muscle (RF) is transferred to the cheek with the vascular pedicle being the facial vessels (FV). The neural in put to the flap is restored by coapting the nerve to the rectus femoris (nRF) to either the marginal mandibular branch (MM) or the ventral ramus of the buccal branch (vrBB) of the facial nerve. *(Lower)* Peripheral reconstruction model showing the transferred muscle reinnervated by either the original nerve or the neighbouring nerve to vastus lateralis (nVL). Also illustrated are the femoral motor nerve root (FMN), and the nerve to the vastus medialis and intermedialis muscles (nVM, VI).



3.4 Results

3.4.1 Facial Reanimation Model – Isometric Force Measurements

Each muscle transfer was subject to 3 episodes of indirect stimulation (using platinum electrodes applied across the supplying motor nerve 1cm proximal to the site of neurorrhaphy). Each episode was 1 second in duration, with a 10V potential applied across the electrodes at 100Hz. A 10 second interval was used between stimulations. The maximal isometric force generated by the muscle (P_o) was measured for each contraction and recorded in grams. The average of these 3 measurements was taken as P_o.

The P_o developed by the 6 month marginal mandibular group (6mm) was 115.19g (stdev 29.03, SEM 11.86), with the 6 month buccal branch group exerting 229.15g (stdev 42.6, SEM 17.40) on average. This difference was significant $p < 0.001$. At 9 months the P_o of marginal mandibular group (9mm) was 137.94g (stdev 21.48, SEM 5.90) compared to 264.38g (stdev 52.6, SEM 14.45) in the buccal branch group (9bb), this finding also being significant $p = 0.002$ (Mann Whitney rank sum test used due to data failing the equal variance test).The increase in P_o seen between time points in both groups was examined, this being significant in the mm group (115.19g vs. 137.94g) $p = 0.001$, but failing to achieve statistical significance in the bb group (229.15g vs. 264.38g) $p = 0.231$. The contralateral rectus femoris muscle served as a control and comparison of the same parameters was undertaken to ensure no difference existed between the groups (ie; the P_o developed by the control muscles of one group was not different to that of another at the same time point). No statistical difference was noted between the mean P_o values of control muscles (c) in the mm and bb groups at either time point (P_o c6mm 1371.8g [stdev 114.34, SEM 46.67] vs. c6bb 1611.8g [stdev 239.29, SEM 97.69] $p = 0.051$, P_o c9mm 1585.7g [stdev 246.89, SEM 100.79] vs. c9bb 1715.5g [stdev 291.58, SEM 119.04] $p = 0.424$), nor was there any significant difference between time points within the groups (P_o 6mm 1371.8g vs 9mm 1585.7g $p = 0.083$, P_o 6bb 1611.8g vs. 9bb 1715.6g $p = 0.51$). The above data is represented graphically in figures 3.4.1 and 3.4.2.

Although no differences were statistically significant between the P_o of the control muscles in any group, in order to reduce differences seen in the P_o developed between transplant groups that could possibly be attributable to variations in the size of the rectus femoris transfers, the P_o was adjusted relative to the muscle mass calculated as force developed per gram of muscle (or weight adjusted specific force [wP_o]). In this way its was felt that a more accurate assessment of the physiological status of the transferred muscle could be made (as opposed to raw force which would not account for differences between the size of the transplants and so could introduce a degree of result bias).

At 6 months post transfer the buccal branch group of transfers produced on average 36.65g force/g muscle (stdev 7.25, SEM 2.96). This contrasted with 24.86 wP_o (stdev 5.16, SEM 2.10) produced by the 6mm group, the difference was statistically significant, $p = 0.009$. At 9 months post transfer, the wP_o generated by both groups of muscle transfers had increased. The 9mm group developed 33.03g force/g muscle (stdev 4.06, SEM 1.66) and the 9bb group 59.41g force/g muscle (stdev 13.67, SEM 5.58). Again, the difference between the groups was statistically significant $p = 0.001$. The difference between the wP_o generated in the 6 month groups was 32.1%, increasing to 44.4% between the 9 month groups. The difference in wP_o between the 2 time points in both the mm and bb groups was significant, $p = 0.012$ and $p = 0.005$ respectively.

In order to evaluate whether the differences seen were due to variance in rectus femoris muscle physiology between the groups (ie; the rabbits in the buccal branch groups developed a greater wP_o within their rectus muscles) the contralateral control rectus femoris muscle wP_o values were compared, so as to act as a control.

wP_o for the control muscles of the 6mm group was 120.52 (stdev 11.93, SEM 4.87) compared to 129.46 (stdev 16.47, SEM 6.72) in the 6bb group. This difference was not significant $p = 0.30$. The wP_o for the 9mm group controls was 143.73 (stdev 29.87, SEM 12.19) and 149.44 (stdev 22.01, SEM 8.98) for the 9bb group, the difference being insignificant $p = 0.714$. The increase in P_o seen between the 2 time points within the bb and mm groups was also insignificant, $p = 0.105$ and $p = 0.108$ respectively. The above data is represented graphically in figures 3.4.3 and 3.4.4.

In addition to examination of the data using group mean values and normalized by those of the contralateral control muscles (acceptance of the differences between the transferred muscles as being a real and significant difference due to no significant difference being found between the control muscle groups), some authors have advocated individually normalizing values by expressing the force produced by the transfer as a percentage of that of the contralateral control muscle [9] [11] [10].

The force developed by the transferred muscles expressed as a percentage of that of the controls was examined. At the 6 month time point the mm group developed on average 8.39% of the P_o of the control contra lateral rectus muscle (cP_o) (stdev 1.98, SEM 0.81) compared to 14.37% (stdev 2.94, SEM 1.20) in the bb group, this difference being statistically significant $p = 0.002$. The 9mm group developed 11.25% (stdev 2.24, SEM 0.91) cP_o and the 9bb group 15.76% (stdev 4.21, SEM 1.72). This difference was again significant $p = 0.043$. The increase in cP_o seen between 6 and 9 months in the mm group (8.39% vs. 11.25%) was significant $p = 0.042$. Although an increase was noted between the 2 time points in the bb group (14.37 vs. 15.76) this difference was not significant $p = 0.394$ (assessed using the Mann Whitney rank sum test due to the data failing to be normally distributed). At the 6 month time point the bb group developed 41.4% greater cP_o than the mm group, although by 9 months this gap had narrowed to 28.6%. This data is represented in figure 3.4.5.

As stated previously wP_o normalizes P_o values adjusting data skew that may arise secondary to variance in the sizes of the muscle transfers, giving an "index" of the physiological force generating capability of the muscle per gram of tissue. The wP_o values of the transferred flaps were compared to those of their respective control muscles, evaluating the physiological performance of the transferred tissue compared to the controls gram for gram (control weight adjusted specific force - cwP_o). The 6mm group cwP_o was 20.78 (stdev 4.52, SEM 1.84), with the 6bb cwP_o being 28.27 (stdev 4.11, SEM 1.67). The difference between the groups was statistically significant $p = 0.013$. At 9 months cwP_o in the mm group was 23.56 (stdev 4.09, SEM 1.67) compared to 39.74 (stdev 7.96, SEM 3.25) for the bb group. This difference was significant $p = 0.001$. A difference in the cwP_o was noted between time points in both groups (mm; 20.78 vs. 23.56, bb; 28.27 vs. 39.74), though this increase was only significant in the bb

Figure 3.4.1: Force developed by muscle transfers in facial reanimation model. The groups are labelled on the x-axis, the force (grams) developed by the transfer on the y-axis. The mean value for each group is given within the column, the SEM value above the respective error bar. Significant values are denoted by an overhead line between the respective groups.

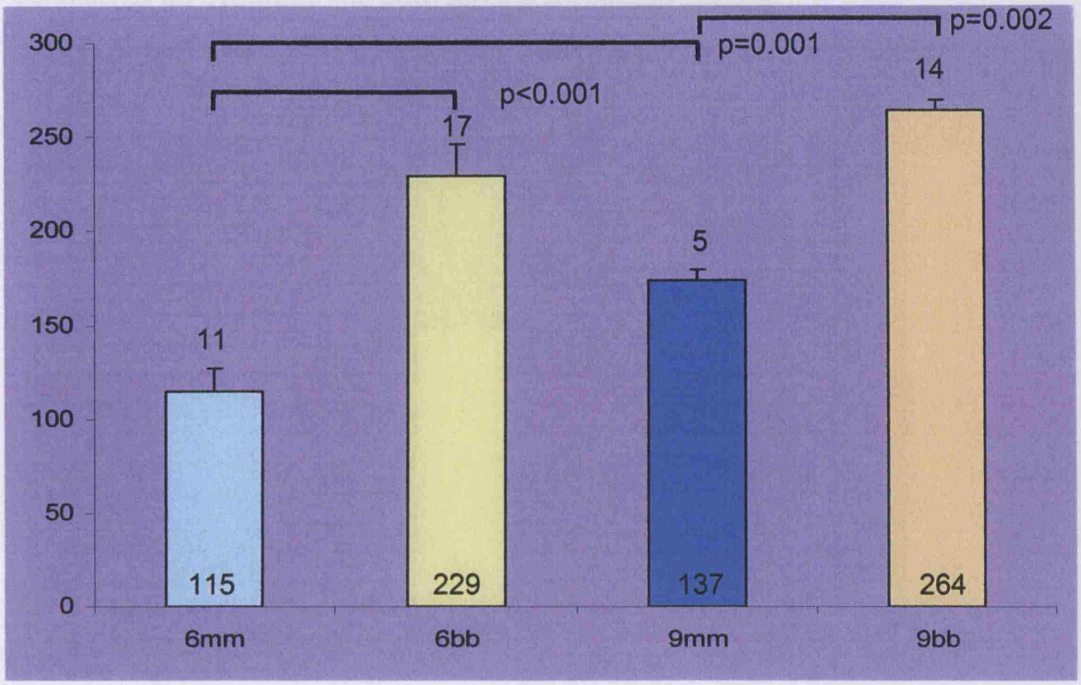


Figure 3.4.2: Force developed by control muscles in facial reanimation model. The groups are labelled on the x-axis, the force (grams) developed by the muscle on the y-axis. The mean value for each group is given within the column, the SEM value above the respective error bar. Significant values are denoted by an overhead line between the respective groups.

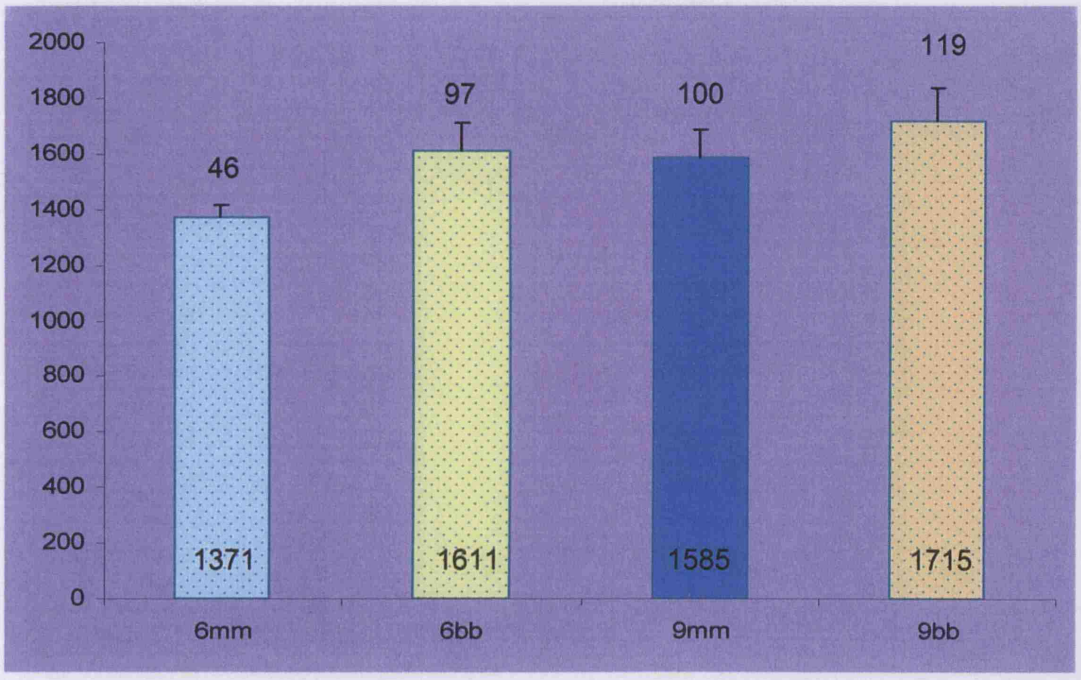


Figure 3.4.3: Weight adjusted force developed by muscle transfers in facial reanimation model. The groups are labelled on the x-axis, the force (grams per gram) developed by the transfer on the y-axis. The mean value for each group is given within the column, the SEM value above the respective error bar. Significant values are denoted by an overhead line between the respective groups.

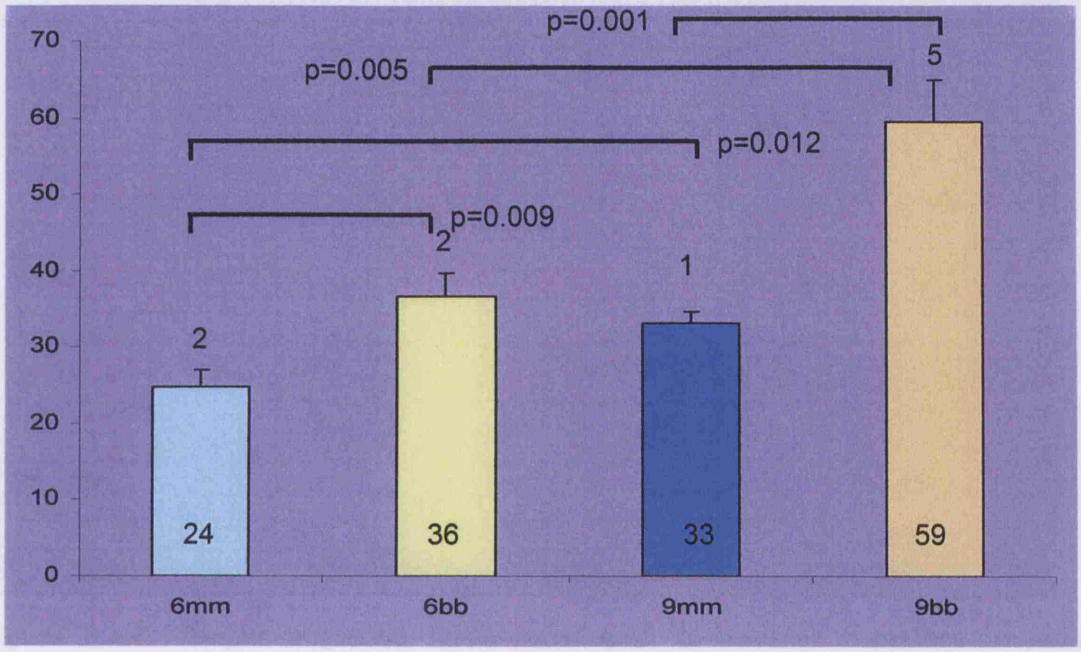


Figure 3.4.4: Weight adjusted force developed by control muscles in facial reanimation model. The groups are labelled on the x-axis, the force (grams per gram) developed by the muscle on the y-axis. The mean value for each group is given within the column, the SEM value above the respective error bar. Significant values are denoted by an overhead line between the respective groups.

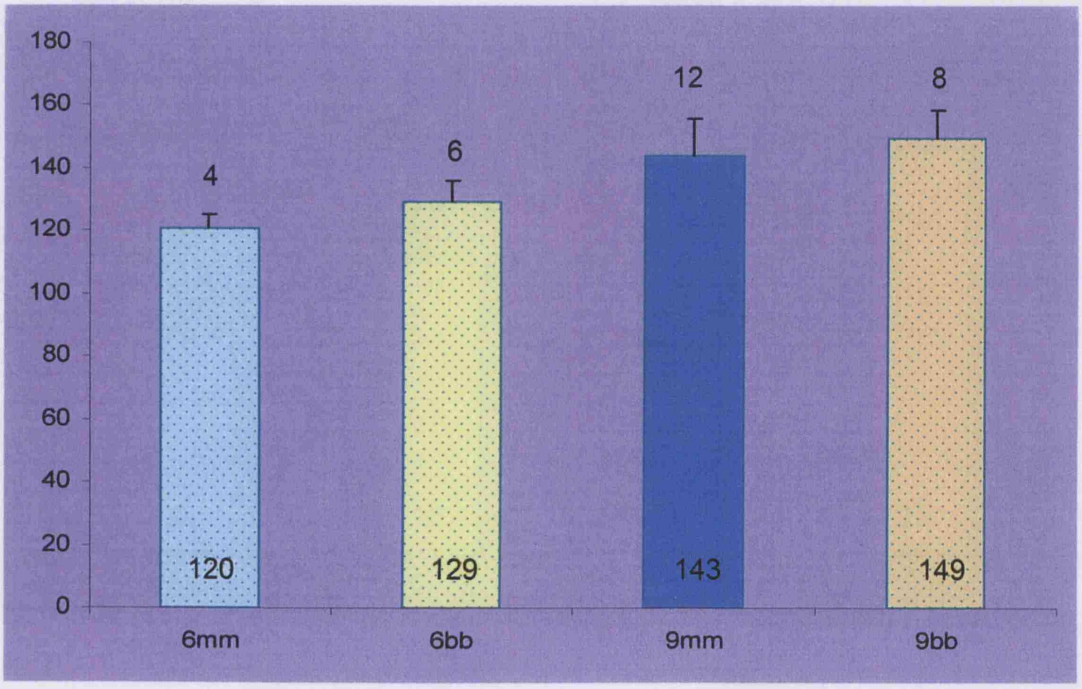


Figure 3.4.5: Force developed by muscle transfers in facial reanimation model expressed as percentage of control muscle. The groups are labelled on the x-axis, the control adjusted force developed by the transfer (%) on the y-axis. The mean value for each group is given within the column, the SEM value above the respective error bar. Significant values are denoted by an overhead line between the respective groups.

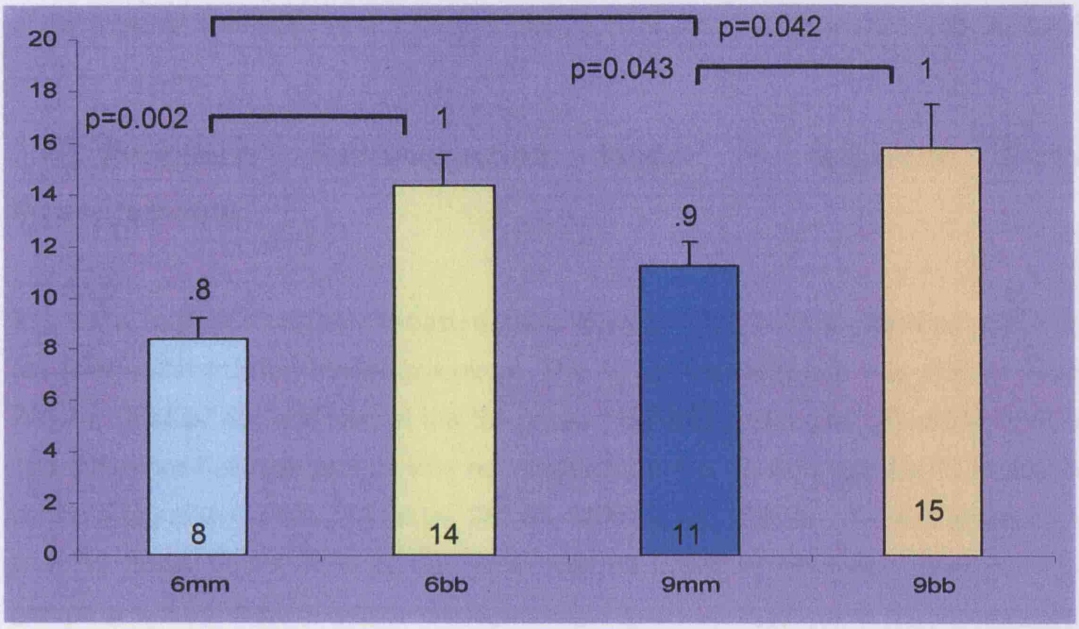
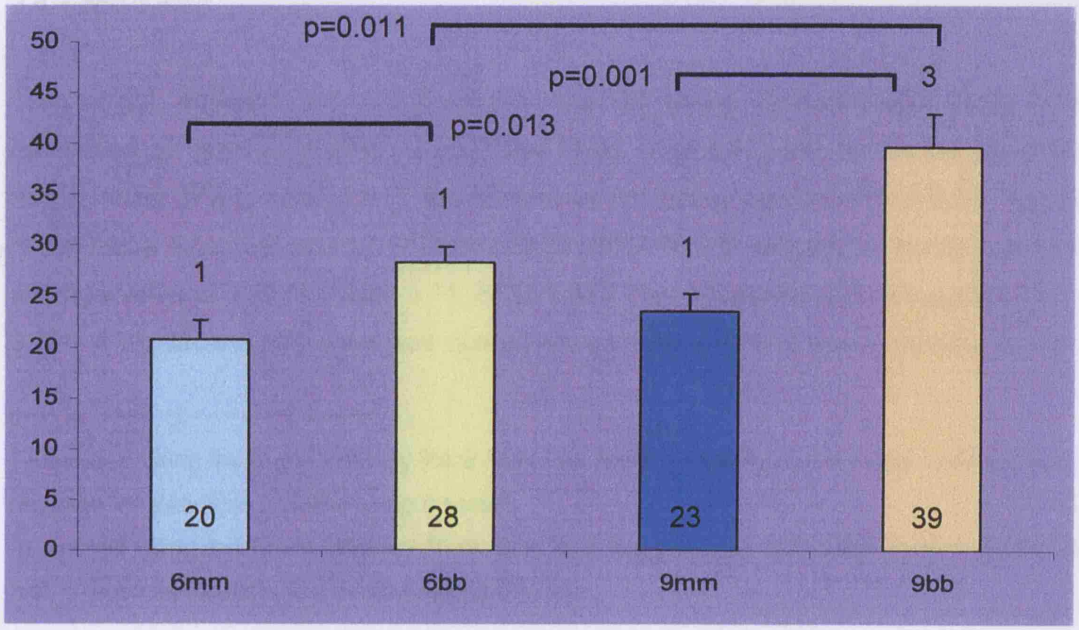


Figure 3.4.6: Weight adjusted force developed by muscle transfers in facial reanimation model expressed as percentage of control muscle. The groups are labelled on the x-axis, the weight and control adjusted force developed by the transfer (%) on the y-axis. The mean value for each group is given within the column, the SEM value above the respective error bar. Significant values are denoted by an overhead line between the respective groups.



group p = 0.011. The difference in cwP_o between the two groups increased from 26.4% at the 6 month time point to 40.71% at 9 months. This data is represented in figure 3.4.6.

3.4.2 Peripheral Reconstruction Model – Isometric Force Measurements

The same muscle stimulation measurements were undertaken at similar time points for the limb reconstruction model groupings. The P_o for the 6rf group was 905.6g (stdev 238.74, SEM 97.46) and that of the 6vl group 1137.09g (stdev 289.04), SEM 118.00). The difference between groups was not significant p = 0.16. At 9 months P_o developed by the rf group was 1198.25g (stdev 243.05, SEM108.96) with that of the vl group being 1140.59 (stdev 66.16, SEM 33.08), there was no statistical difference between these groups p = 0.66. Neither group displayed any significant increase in P_o between time points (p = 0.076 for the rf groups, p = 1.00 for the vl groups[2]). The P_o developed by the control rectus femoris muscles did not demonstrate any significant difference between groupings at 6 months (rf; 1441.97g [stdev 309.08, SEM 126.18], vl; 1432.06g [stdev 144.57, SEM 64.65] p = 0.94) or 9 months (rf; 1916g [stdev 306.52, SEM 76.23], vl; 1800.82 [stdev 152.46, SEM 76.23] p = 0.90). Both groups demonstrated an increase in P_o between time points (rf; p = 0.031, vl; p = 0.008[3]). This data is represented in figures 3.4.7 and 3.4.8.

The weight adjusted measurements for the 6rf group demonstrated 84.9g force developed per gram of muscle tissue (stdev 11.94, SEM 4.87). wP_o for the 6vl group was 98.17 (stdev 19.77, SEM 8.07), this difference not being significant p = 0.19. The 9rf group had a mean wP_o of 113.49 (stdev 23.37, SEM 10.45), with the 9vl group having an average value of 118.71 (stdev 3.74, SEM 1.87). This difference was not significant p = 0.73[2]. A significant difference was noted between the wP_o at 6 and 9 months in the rf

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[2] Assessed using the Mann Whitney Rank Sum test due the data failing the equal variance test, a requisite for statistical analysis using a t-test.

[3]Assessed using the Mann Whitney Rank Sum test due the data failing the normal distribution test, a requisite for statistical analysis using a t-test.

group (84.90 vs. 113.49 respectively) p = 0.027, though this was not seen in the vl groups (98.17 vs. 118.71 respectively) p = 0.079. The control rectus femoris muscle wP_o values did not show any significant difference between groups at 6 months (rf; 122.36 [stdev 31.56, SEM 12.88], vl; 118.86 [stdev 9.40, SEM 4.20], p = 0.81) or at 9 months (rf; 149.38 [stdev 18.09, SEM 8.09, vl; 146.77 [stdev 12.05, SEM 6.02], p = 0.073[3]). An increase in wP_o was seen between the 2 time points in both the rf and vl groups (122.36 vs. 149.38 and 118.86 vs. 146.77 respectively), the difference was however only significant in the vl group p = 0.006. This data is represented in figures 3.4.8 and 3.4.9.

The cP_o values for the 6 month groups were 63.65 (stdev 14.39, SEM 5.87) for the rf group, and 73.34 (stdev 14.28, SEM 5.84) for the vl group, the difference not being statistically significant p = 0.29. The 9rf group demonstrated force production of 62.33% (stdev 4.78, SEM 1.95) of that of the control muscle, the 9vl group 63.46 (stdev 2.70, SEM 1.10), with no significant difference between the 2 groups, p = 0.68. Both groups failed to demonstrate an increase in force production compared to the control muscle between the 6 and 9 month time points (rf; 63.65 vs. 62.33, vl; 73.34 vs. 63.46), indeed the vl group displayed a net decrease in relative force production, however these differences were not significant (rf; p = 0.84, vl; p = 0.21). This data is represented in figure 3.4.11.

The wcP_o value for the 6rf group was 73.81 (stdev 23.83, SEM 9.73), compared to 77.28 (stdev 11.06, SEM 4.94) in the 6vl group. No significant difference was found p = 0.77. The wcP_o developed by the 9rf group was 76.08 (stdev 11.06, SEM 4.94) compared to 81.31 (stdev 7.61, SEM 3.80) seen in the 9vl group. No difference was found between these groups p = 0.52, nor was any difference noted between time periods in either group (6rf 73.81 vs. 9rf 76.08 p = 0.85, 6vl 77.28 vs. 9vl 81.31 p = 0.55). This data is represented in figure 3.4.12.

Figure 3.4.7: Force developed by muscle transfers in peripheral reconstruction model. The groups are labelled on the x-axis, the force (grams) developed by the transfer on the y-axis. The mean value for each group is given within the column, the SEM value above the respective error bar. Significant values are denoted by an overhead line between the respective groups.

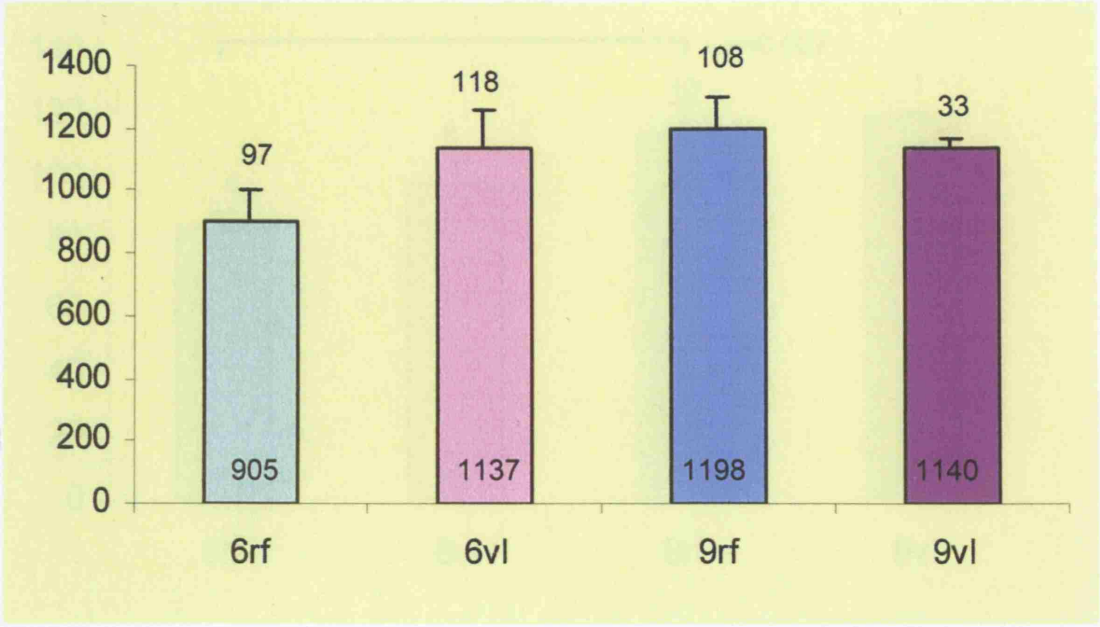


Figure 3.4.8: Force developed by control muscles in peripheral reconstruction model. The groups are labelled on the x-axis, the force (grams) developed by the muscle on the y-axis. The mean value for each group is given within the column, the SEM value above the respective error bar. Significant values are denoted by an overhead line between the respective groups.

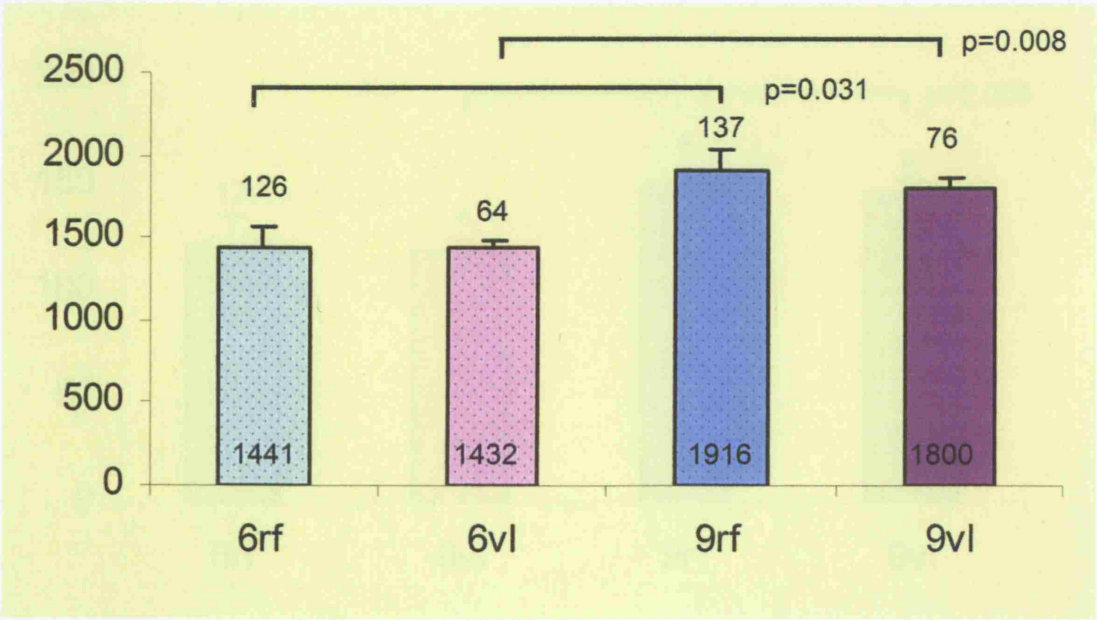


Figure 3.4.9: Weight adjusted force developed by muscle transfers in peripheral reconstruction model. The groups are labelled on the x-axis, the force (grams per gram) developed by the transfer on the y-axis. The mean value for each group is given within the column, the SEM value above the respective error bar. Significant values are denoted by an overhead line between the respective groups.

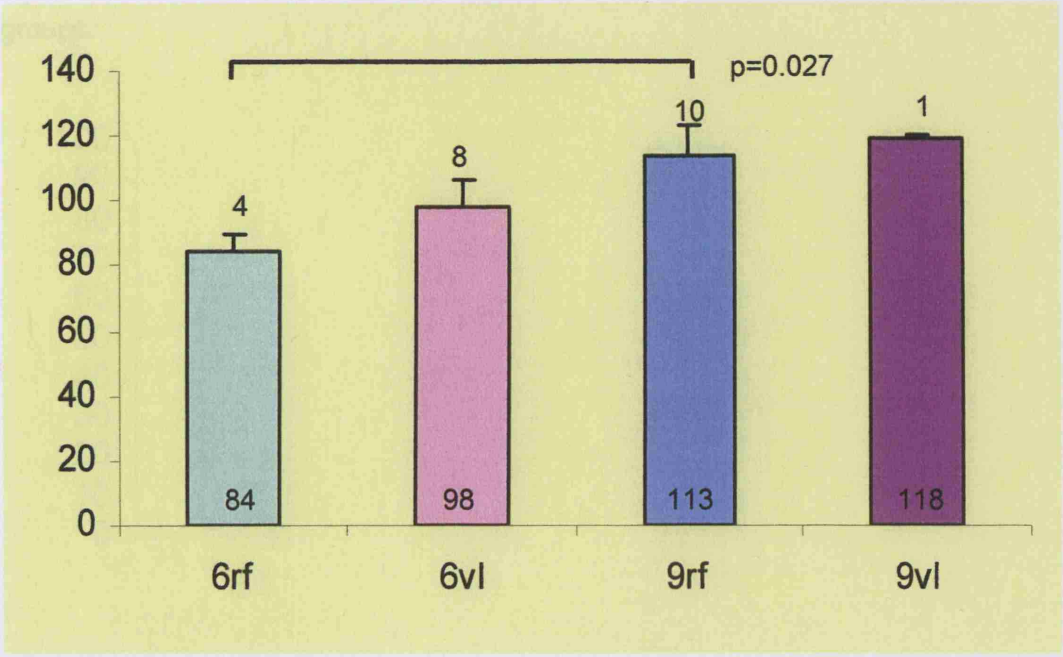


Figure 3.4.10: Weight adjusted force developed by control muscles in peripheral reconstruction model. The groups are labelled on the x-axis, the force (grams per gram) developed by the tra on the y-axis. The mean value for each group is given within the column, the SEM value above the respective error bar. Significant values are denoted by an overhead line between the respective groups.

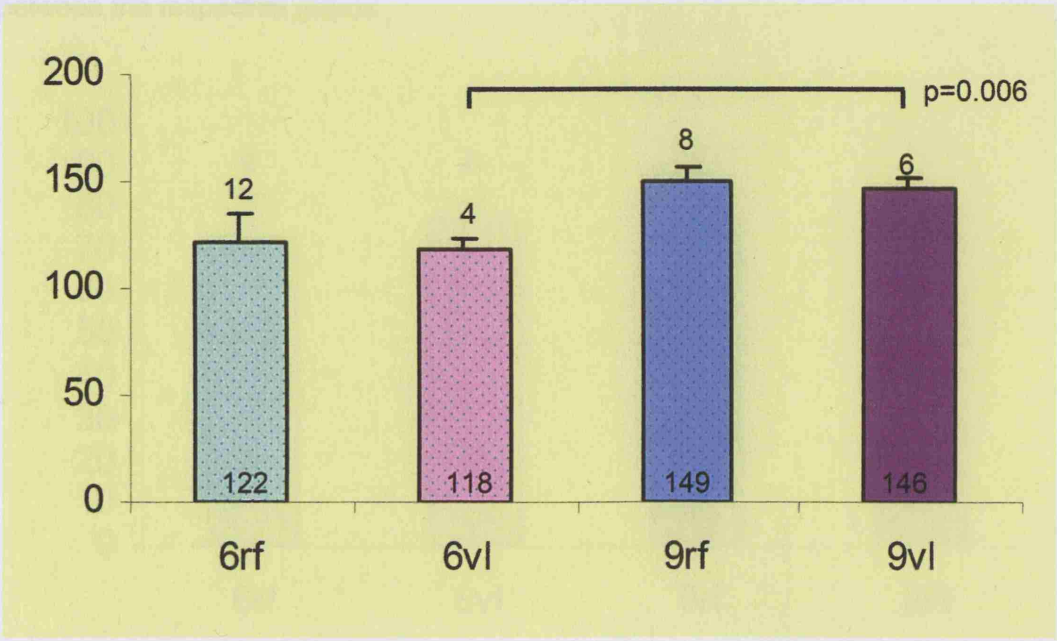


Figure 3.4.11: Force developed by muscle transfers in peripheral reconstruction model expressed as percentage of control muscle. The groups are labelled on the x-axis, the control adjusted force developed by the transfer (%) on the y-axis. The mean value for each group is given within the column, the SEM value above the respective error bar. Significant values are denoted by an overhead line between the respective groups.

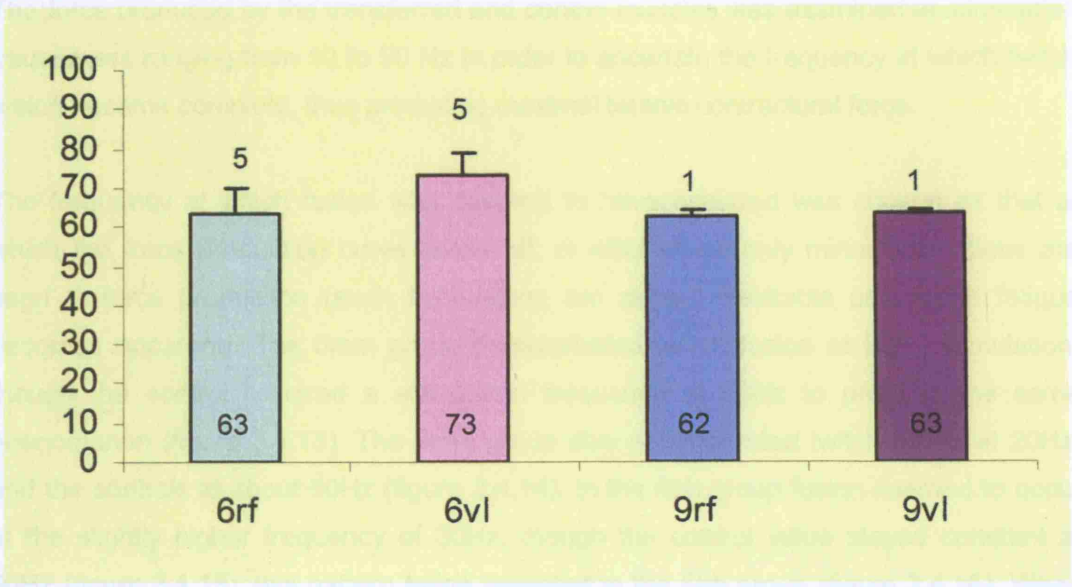
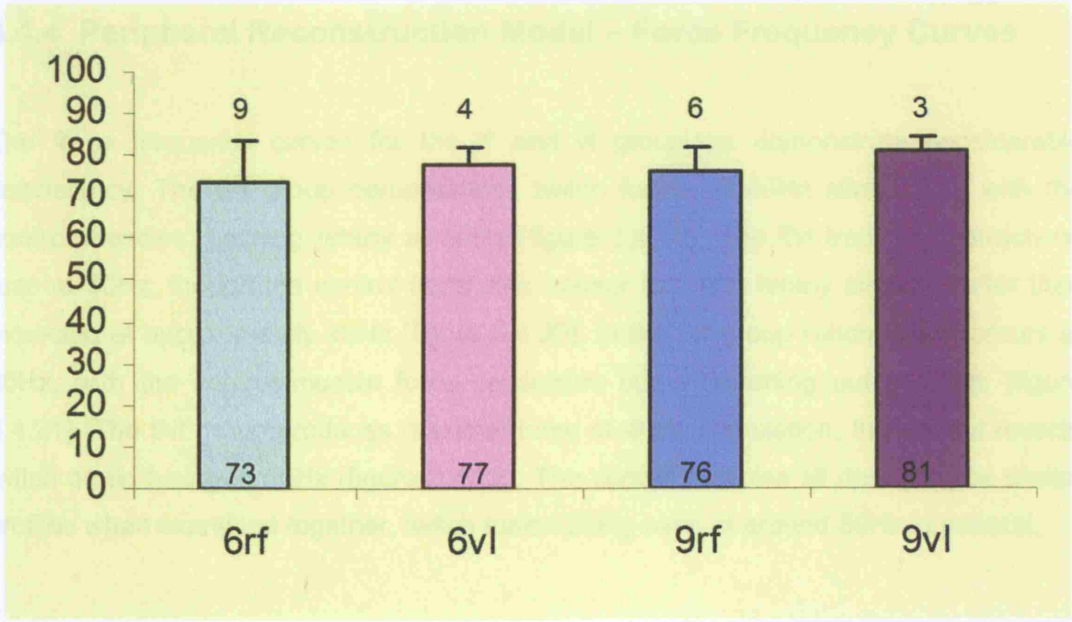


Figure 3.4.12: Weight adjusted force developed by muscle transfers in peripheral reconstruction model expressed as percentage of control muscle. The groups are labelled on the x-axis, the weight and control adjusted force developed by the transfer (%) on the y-axis. The mean value for each group is given within the column, the SEM value above the respective error bar. Significant values are denoted by an overhead line between the respective groups.



3.4.3 Facial Reanimation Model – Force Frequency Curves

The force produced by the transferred and control muscles was examined at stimulation frequencies ranging from 10 to 90 Hz in order to ascertain the frequency at which twitch fusion became complete, thus producing maximal tetanic contractural force.

The frequency at which fusion was deemed to have occurred was chosen as that at which the force production curve levels off, or after which only minor fluctuations are seen in force production (such fluctuations are almost inevitable as muscle fatigue becomes apparent). The 6mm group demonstrated twitch fusion at 20Hz stimulation, though the control required a stimulation frequency of 50Hz to produce the same phenomenon (figure 3.4.13). The 9mm group also demonstrated twitch fusion at 20Hz, and the controls at about 50Hz (figure 3.4.14). In the 6bb group fusion seemed to occur at the slightly higher frequency of 30Hz, though the control value stayed constant at 50Hz (figure 3.4.15), this pattern being repeated in the 9bb group (figure 3.4.16). When grouped together the control muscle force – frequency profiles all produce similar curves with twitch fusion occurring at 50Hz (figure3.4.18). As a contrast to this, the operated muscles demonstrate a sequential fusion frequency between the mm and bb groups, with the mm groupings producing twitch fusion at 20Hz and the bb groupings force production only leveling off at stimulating frequencies higher than 30Hz (figure 3.4.17).

3.4.4 Peripheral Reconstruction Model – Force Frequency Curves

The force frequency curves for the rf and vl groupings demonstrate considerable consistency. The 6rf group demonstrates twitch fusion at 40Hz stimulation, with the control muscles reaching tetany at 50Hz (figure 3.4.19). The 6vl transfer contractions fuse at 30Hz, though the control flaps also appear to reach tetany slightly earlier than expected at approximately 40Hz (figure 3.4.20). In the 9rf group twitch fusion occurs at 30Hz, with the control muscle force production curve flattening out at 50Hz (figure 3.4.21). The 9vl group produces maximal force at 40Hz stimulation, the control muscle twitch again fusing at 50Hz (figure 3.4.22). The control muscles all demonstrate similar profiles when examined together, twitch fusion being seen at around 50Hz in general,

Figure 3.4.13: Force frequency curves for 6mm group transfer and control muscles. Stimulation frequency is given on the x-axis (Hz), force developed (grams) by the transfer (left side) and control muscle (right side) on the y–axis. The control muscle values are the dark green trace.

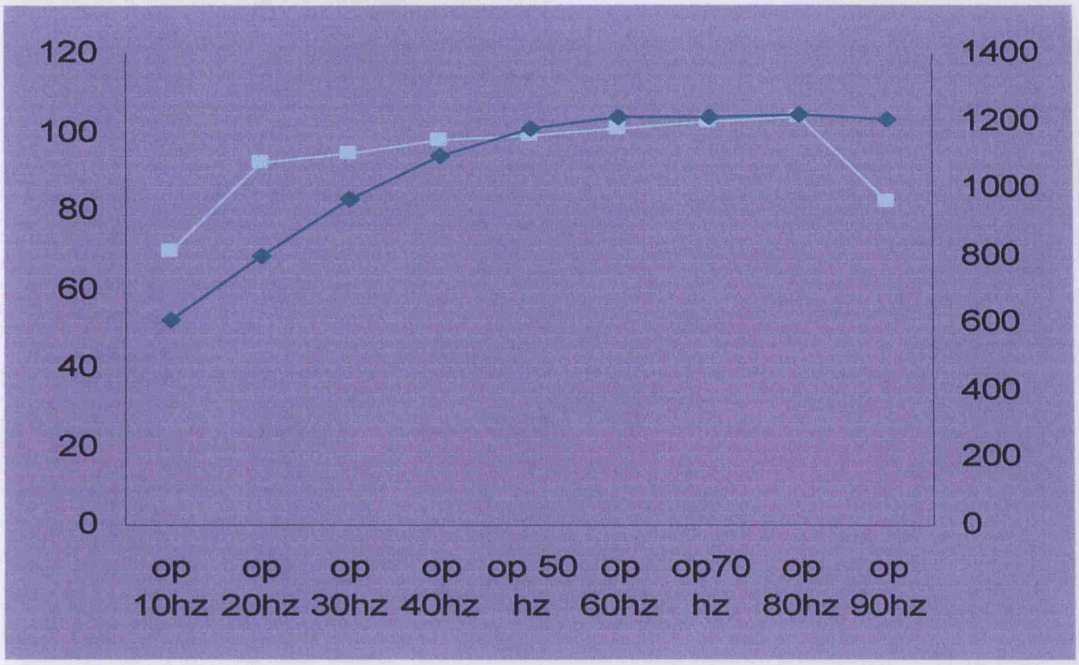


Figure 3.4.14: Force frequency curves for 6bb group transfer and control muscles. Stimulation frequency is given on the x-axis (Hz), force developed (grams) by the transfer (left side) and control muscle (right side) on the y–axis. The control muscle values are the dark green trace.

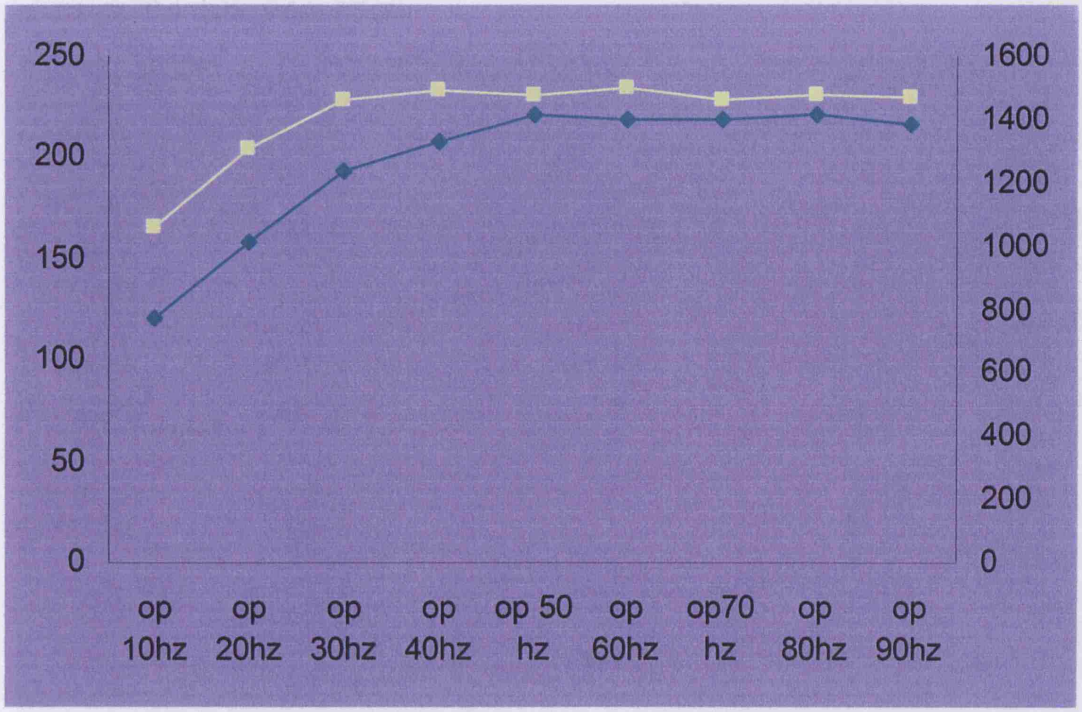


Figure 3.4.15: Force frequency curves for 9mm group transfer and control muscles. Stimulation frequency is given on the x-axis (Hz), force developed (grams) by the transfer (left side) and control muscle (right side) on the y–axis. The control muscle values are the dark green trace.

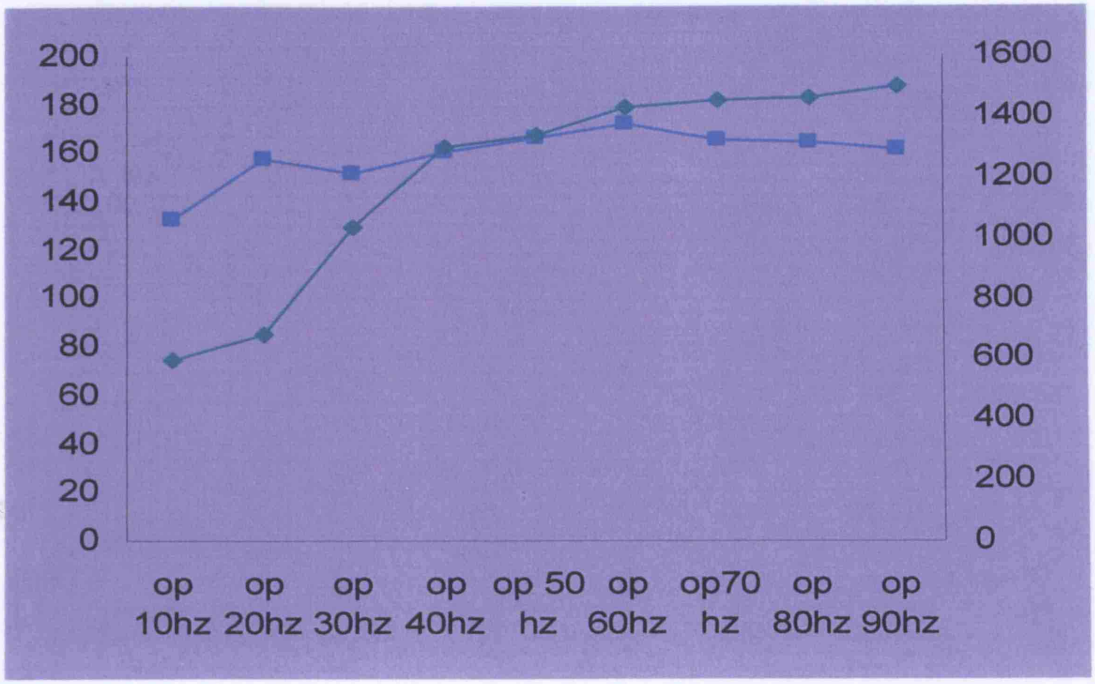


Figure 3.4.16: Force frequency curves for 9bb group transfer and control muscles. Stimulation frequency is given on the x-axis (Hz), force developed (grams) by the transfer (left side) and control muscle (right side) on the y–axis. The control muscle values are the dark green trace.

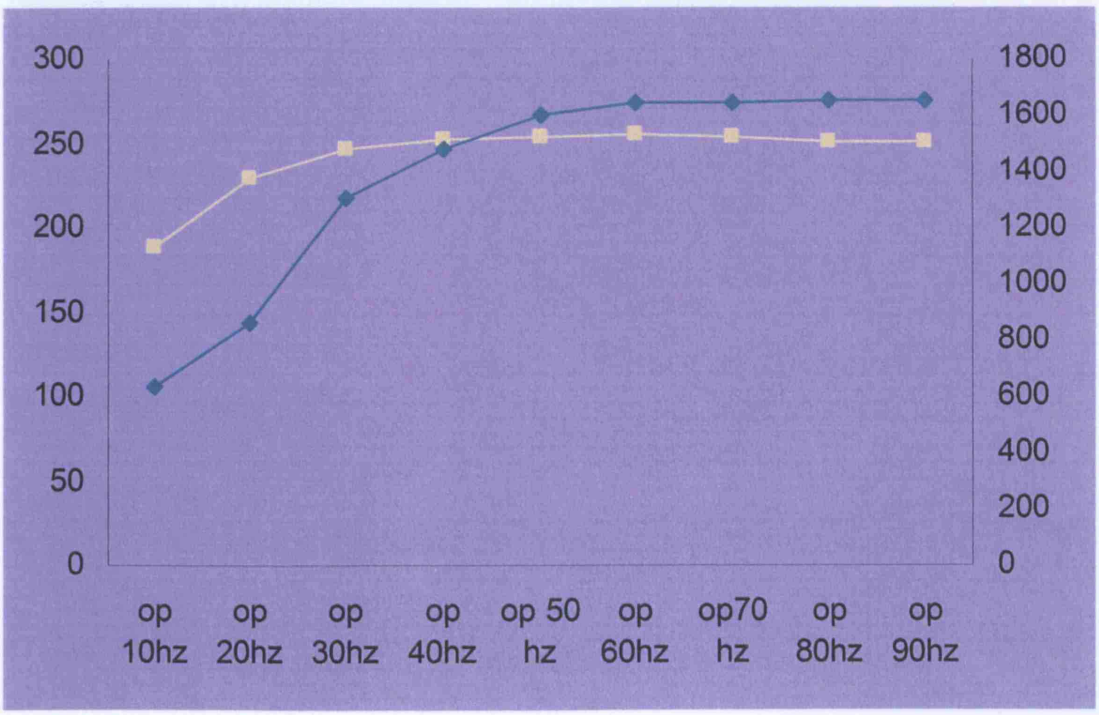


Figure 3.4.17: Force frequency curves for the facial reanimation group transfer muscles. Stimulation frequency is given on the x-axis (Hz), force developed (grams) by the transferred muscles on the y–axis. The identity of the individual groups is given in the legend on the right of the graph.

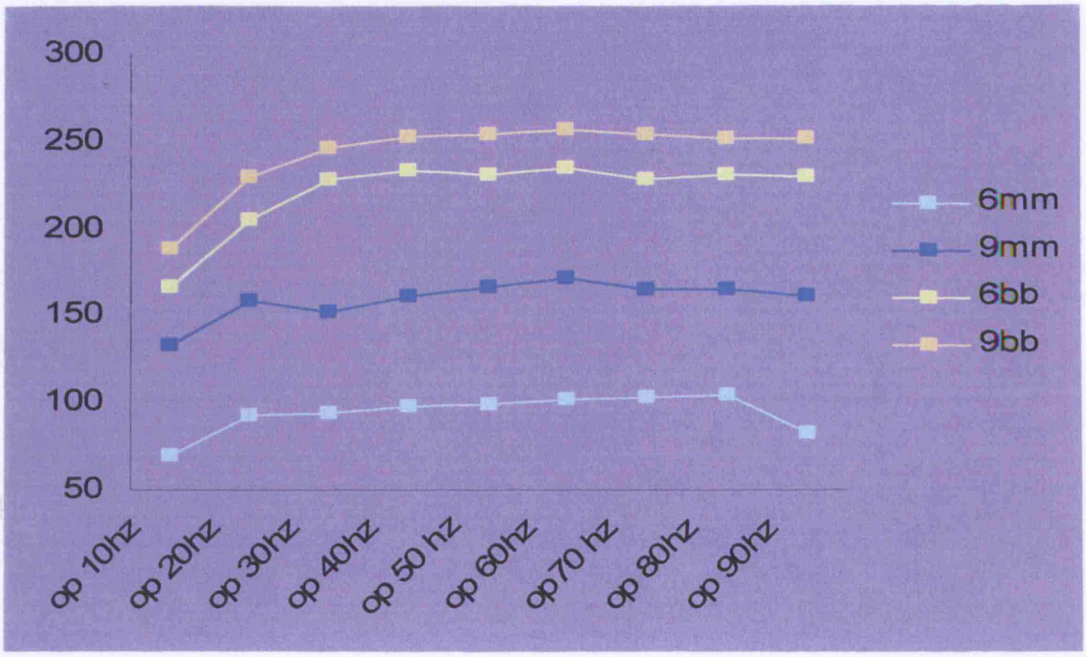


Figure 3.4.18: Force frequency curves for the facial reanimation group control muscles. Stimulation frequency is given on the x-axis (Hz), force developed (grams) by the control muscles on the y–axis. The identity of the individual groups is given in the legend on the right of the graph.

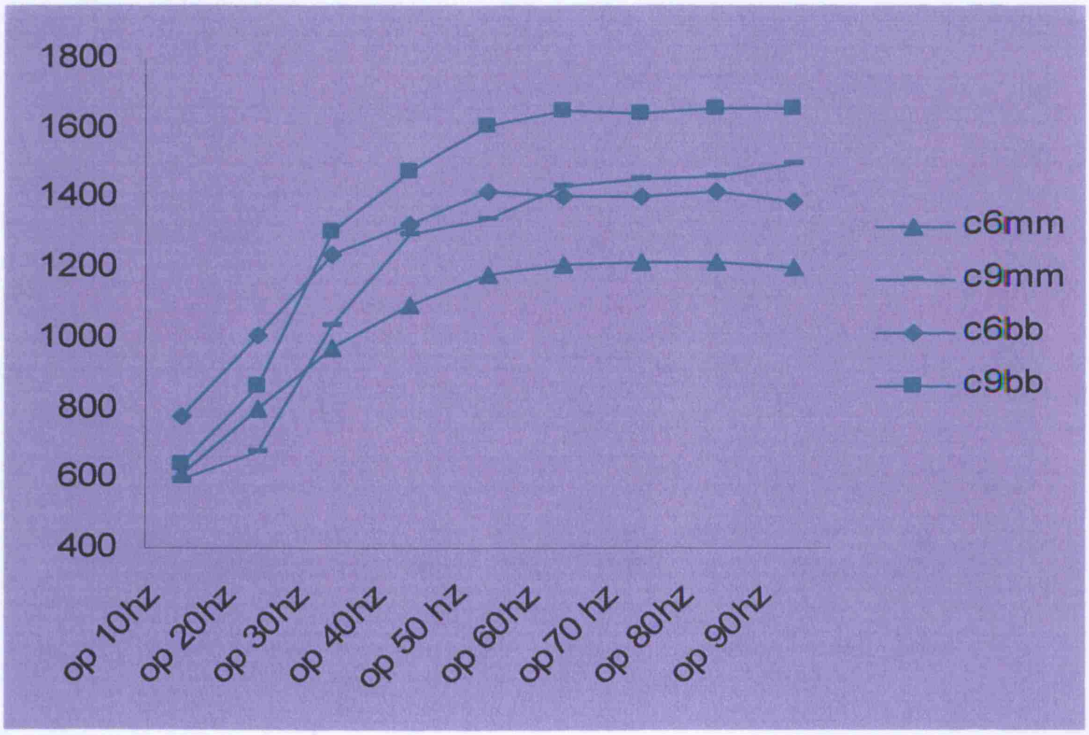


Figure 3.4.19: Force frequency curves for 6rf group transfer and control muscles. Stimulation frequency is given on the x-axis (Hz), force developed (grams) by the transfer (left side) and control muscle (right side) on the y–axis. The control muscle values are the dark green trace.

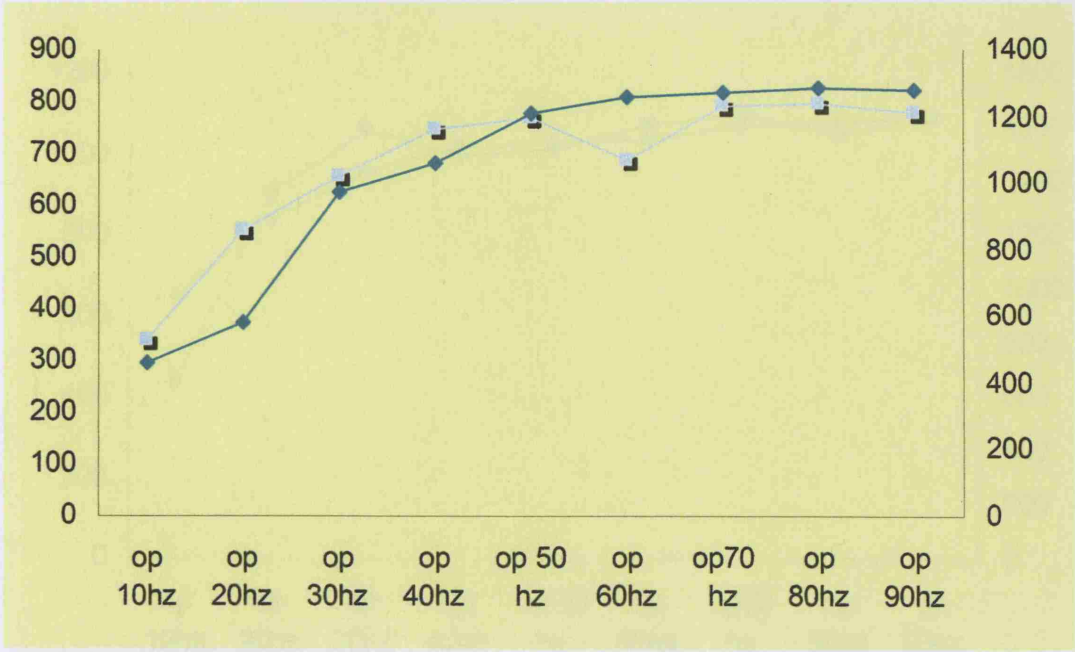


Figure 3.4.20: Force frequency curves for 6vl group transfer and control muscles. Stimulation frequency is given on the x-axis (Hz), force developed (grams) by the transfer (left side) and control muscle (right side) on the y–axis. The control muscle values are the dark green trace.

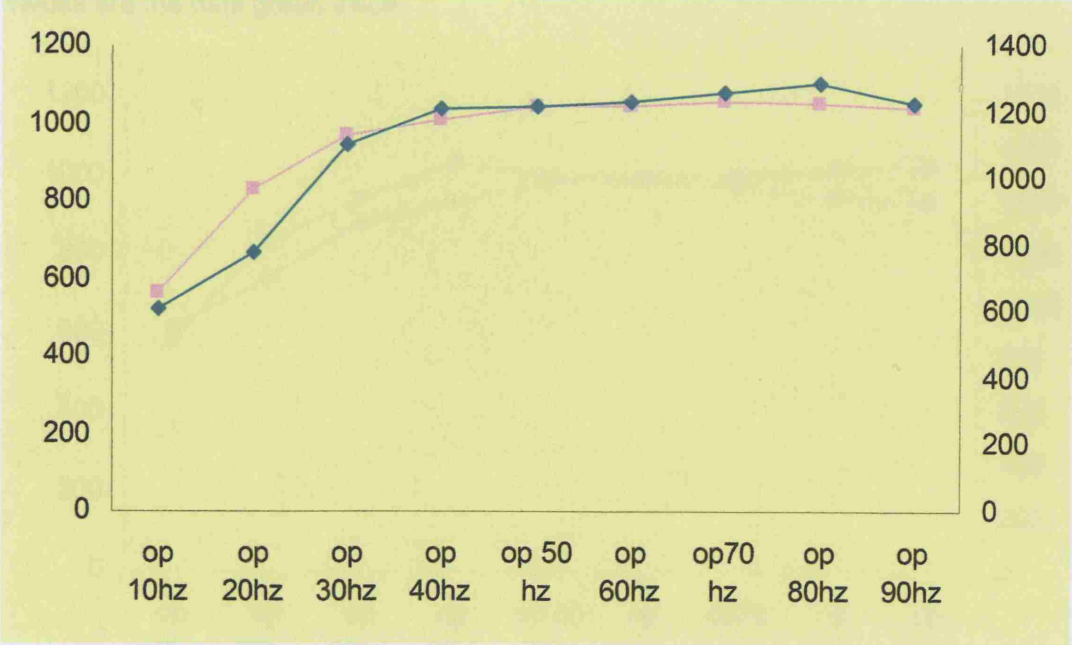


Figure 3.4.21: Force frequency curves for 9rf group transfer and control muscles. Stimulation frequency is given on the x-axis (Hz), force developed (grams) by the transfer (left side) and control muscle (right side) on the y–axis. The control muscle values are the dark green trace.

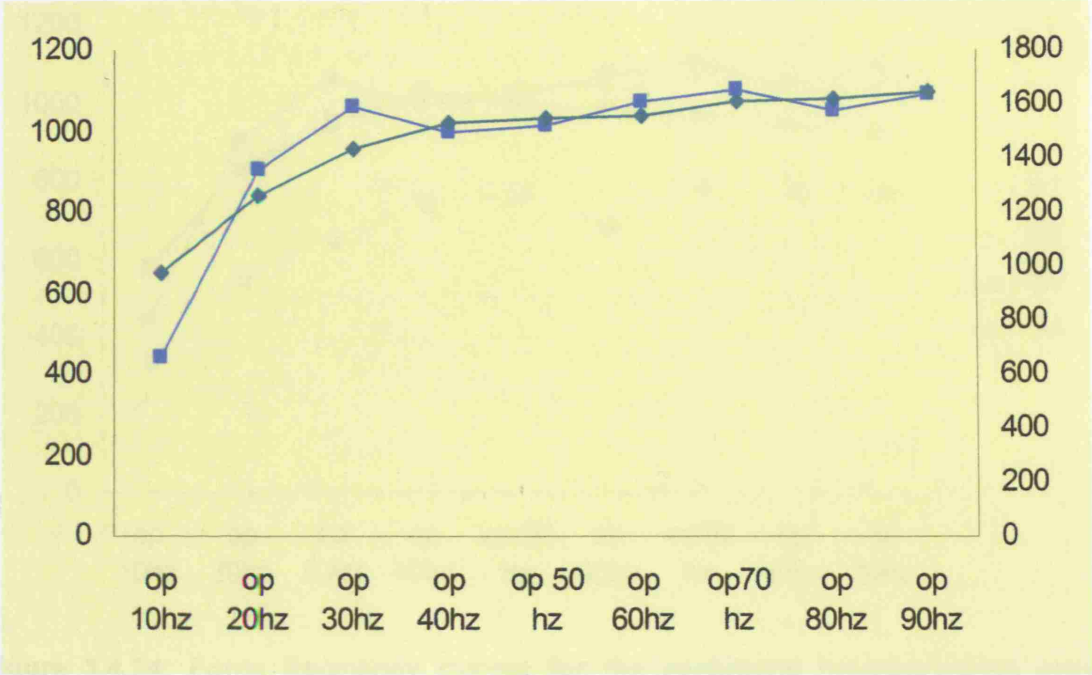


Figure 3.4.22: Force frequency curves for 9vl group transfer and control muscles. Stimulation frequency is given on the x-axis (Hz), force developed (grams) by the transfer (left side) and control muscle (right side) on the y–axis. The control muscle values are the dark green trace.

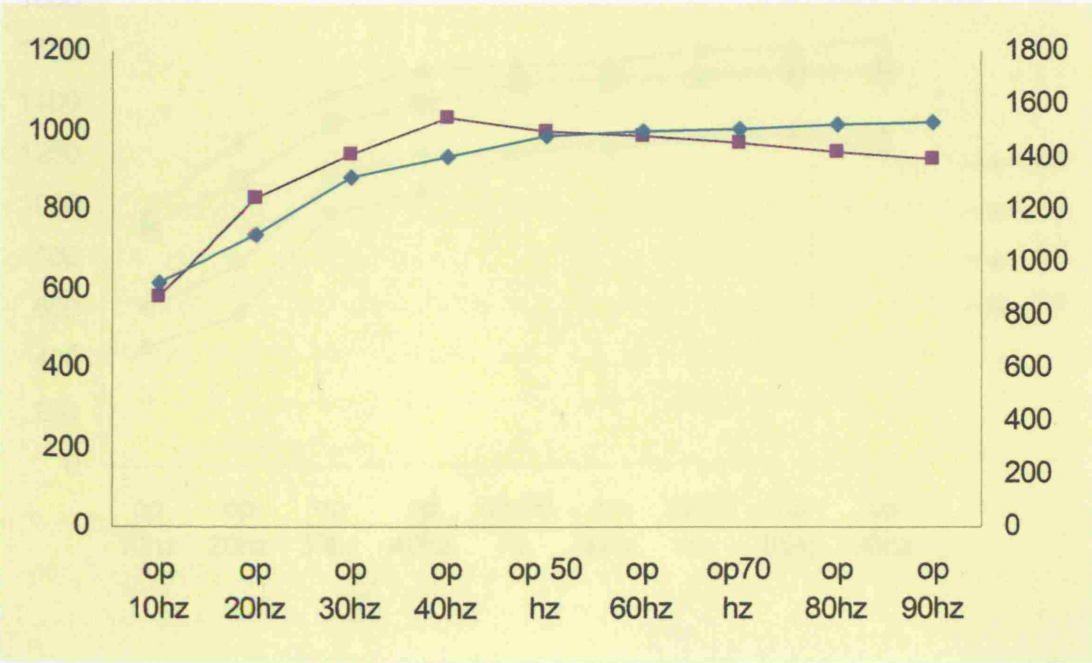


Figure 3.4.23: Force frequency curves for the peripheral reconstruction group transfer muscles. Stimulation frequency is given on the x-axis (Hz), force developed (grams) by the transferred muscles on the y–axis. The identity of the individual groups is given in the legend on the right of the graph.

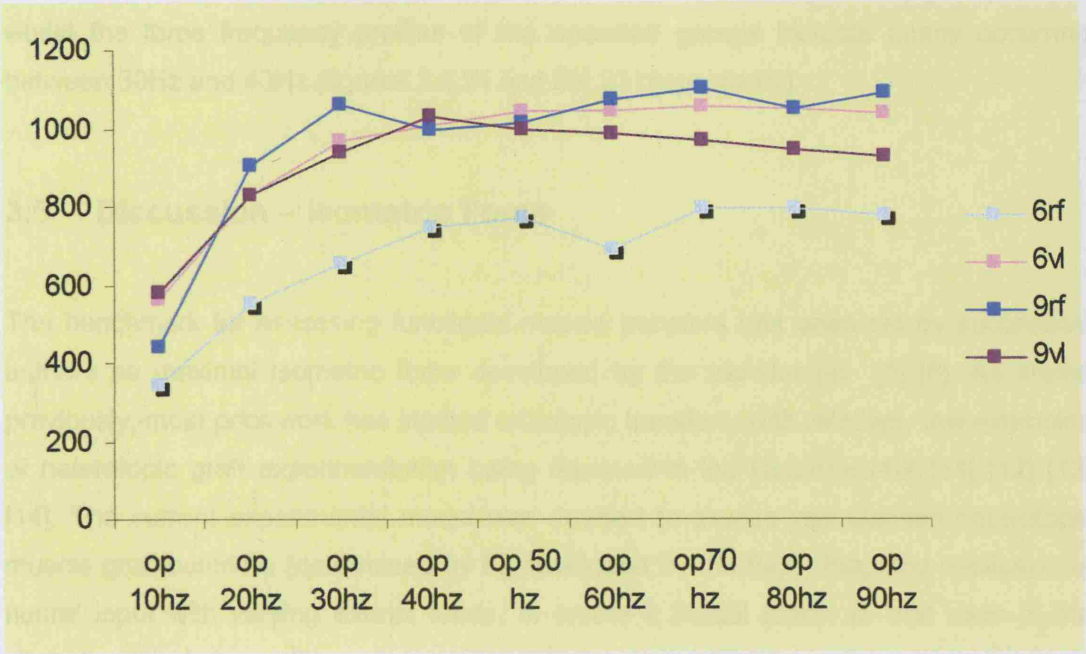
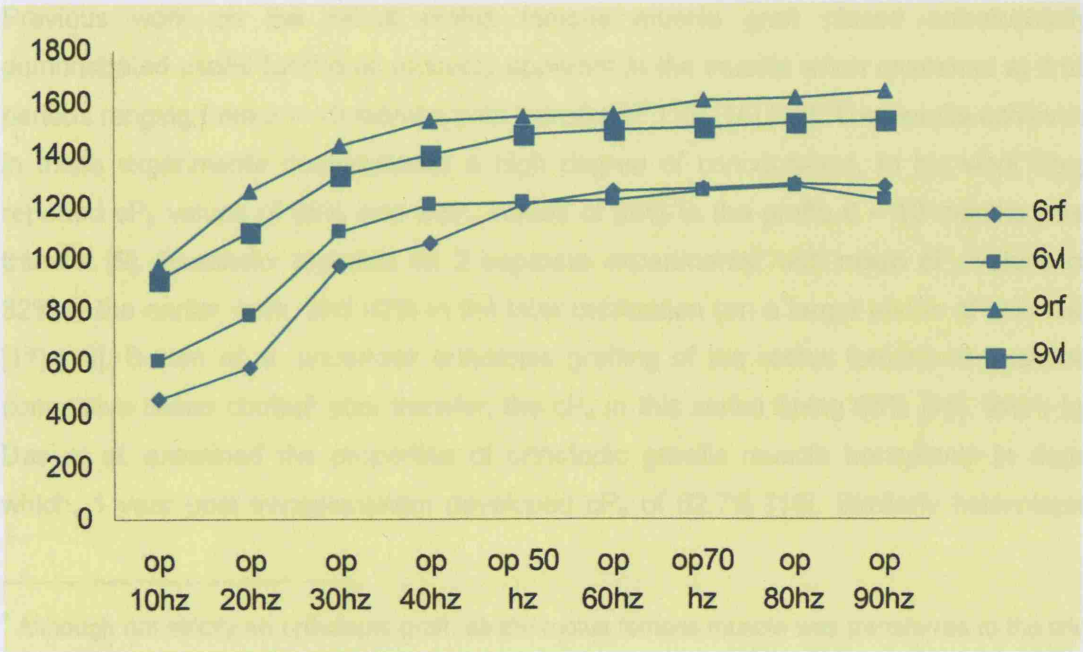


Figure 3.4.24: Force frequency curves for the peripheral reconstruction group control muscles. Stimulation frequency is given on the x-axis (Hz), force developed (grams) by the control muscles on the y–axis. The identity of the individual groups is given in the legend on the right of the graph.



whilst the force frequency profiles of the operated groups indicate tetany occurring between 30Hz and 40Hz (figures 3.4.24 and 3.4.23 respectively).

3.5 Discussion – Isometric Force

The benchmark for assessing functional muscle transfers has been set by successive authors as maximal isometric force developed by the transfer [4] [5] [6]. As stated previously, most prior work has studied orthotopic transfers, with relatively few examples of heterotopic graft experimentation being reported in the literature [10] [11] [12] [13] [14]. The current experimental model was devised to assess vascularised heterotopic muscle graft outcome (determined by P_o developed by the flap) following restitution of neural input with varying axonal loads, to create a model closer to that seen in the clinical setting (where all transfers are by definition heterotopic).

3.5.1 Peripheral Reconstruction Model

Previous work on the rabbit rectus femoris muscle graft placed orthotopically demonstrated useful functional recovery apparent in the muscle when examined at time periods ranging from 3 – 10 months post transfer[9][4] [15] [16] [17]. The results achieved in these experiments demonstrated a high degree of concordance. In his work Frey reported cP_o values of 55% and wcP_o values of 68% in the grafts 6 – 10 months post transfer [9]. Guelinckx reported on 2 separate experiments, with mean cP_o values of 32% in the earlier work, and 62% in the later publication (on a larger series of animals) [17] [16]. Burton *et al.* undertook orthotopic grafting of the rectus femoris to examine connective tissue content post transfer, the cP_o in this series being 65% [15]. Work by Das *et al.* examined the properties of orthotopic gracilis muscle transplants in dogs which, 1 year post transplantation developed cP_o of 62.7% [18]. Similarly heterotopic

[4] Although not strictly an orthotopic graft, as the rectus femoris muscle was transferred to the site of the contra lateral rectus femoris, it is assumed that the neural supply to the muscle will be composed of a similar axonal number of equivalent sizes, and will therefore be considered as an orthotopic graft for these purposes.

latissimus dorsi muscles (transferred to the rectus femoris muscle bed in the New Zealand White rabbit) undertaken by Guelinckx *et al.* developed approximately 70% maximal tetanic force of the control latissimus dorsi [19].

Of interest in the latter 2 studies is the fact that the transferred muscle grafts developed weight or muscle cross sectional area adjusted force values of 100% of that of the control. These values have been unachievable in rectus femoris grafts. Both of the transferred muscles in these studies were parallel fibered muscles, with long muscle fiber lengths. A suggestion put forward explaining the difference between these muscles and the rectus femoris is that the neuromuscular junctions in the parallel fibered muscles all lie within a close distance of the hilum (resulting in complete reinnervation of all surviving muscle fibers), whereas in the pennate muscle they are dispersed throughout its length resulting in prolonged reinnervation times with an attendant increased population of denervated fibers distally [19]. An alternative explanation to this observed phenomenon (taking into account the fact that the transplants in these studies still only managed to develop 60% – 70% of the force of the control) is that the average atrophy of individual fibers is less in the rectus femoris and so the muscle retains more of its overall mass equating to a decrease in the force per gram of muscle[5]. Regardless of the differential between parallel and pennate muscles in terms of wcP_o values, none of the published studies have managed to approach 100% of the force of the control muscle following transfer, with most quoting rates of approximately 60% recovery of function [9] [16] [14] [17] [18] [15].

The above data is consistent with the findings in the 6rf group from this study which developed a cP_o of 63.65%. The degree of consistency between results obtained by differing authors lends weight to the collective findings and strongly suggests that when a muscle graft is placed (with microneurovascular repair) orthotopically, the maximum recovery achievable using current techniques is somewhere between 50 and 70%[6].

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[5] In the study by Guelinckx *et al.* mean transplanted latissimus dorsi fibre size decreased by 63% compared to 43% in rectus femoris transplants.

[6] The wcP_o value may be somewhat higher than this as it takes into account any decrease in muscle size experienced by the flap relative to the control, and looks purely at the physiological capability of a set mass of muscle tissue.

Clinical data suggests that the gracilis muscle, when used to restore forearm flexor function, can achieve 50% of the power of the normal arm in optimum cases [20], however comparison with the above situations is not entirely valid as isolated muscle function cannot be examined in the normal arm (ie; flexor digitorum profundus function alone without the effect of superficialis and pollicis longus), nor is there any data readily available on the performance of the gracilis muscles prior to transplantation. What is apparent however, is that there is a significant discrepancy in performance between the normal and reconstructed sides (it must also be noted that mechanical inefficiency due to tendon adhesion, joint instability and the like will all contribute to force dissipation in the operated limb).

One possible reason for the decrease in function seen post transfer is a residual population of denervated cells. This possibility has been examined in rat muscle where its motor nerve has been divided and varying proportions of it coapted back to the muscle [21]. Reduced muscle specific force resulted from this action, and histological investigation confirmed the presence of a residual population of denervated muscle fibres. If this situation is the reason behind the decreased performance seen post transfer then the denervation may be the result of neuronal escape at the neurorrhaphy or the failure of axonal bridging as a result of scarring at the site of coaptation. An increase in the number of axons pre-neurorrhaphy would make up for axonal loss across the neurorrhaphy and potentially eliminate the denervated population of muscle cells. Preliminary work carried out prior to the commencement of the main experiment determined that the axonal mass of the vastus lateralis muscle motor nerve was approximately twice that of the rectus femoris muscle.

The 6vl group displays a trend for mildly superior results over the 6rf group in terms of P_o, wP_o, cP_o and wcP_o, though none of these findings achieved significance. By 9 months any difference between the groups has disappeared. The noted increase in raw force or P_o developed between the 2 time points in the groupings (a trend, not significant) can be explained by a mirrored phenomenon in the control muscles (significant), and probably simply represents growth in the muscle mass as the rabbit matures. Increases in the wP_o of the rf and vl groupings between time points of 25% and

17% respectively[7] were paralleled by 18% and 19% increases[8] respectively in the control muscles. This increase in physiological capability of the muscle is likely to be the result of changes in the myocytes with maturity[a1] and not the result of changes in innervation status in the transferred muscles between 6 and 9 months. This theory is backed up by the individually normalized muscle data (cP_o and wcP_o) which demonstrates that any increases noted between time points within the groupings in terms of P_o and wP_o are eradicated. These findings concur with Geulinckx *et al.* who found that at 90 days post orthotopic transfer no difference was found between direct and indirect stimulation of the muscle transplants (indicating that all non refractory muscle fibers were now reinnervated) and that wP_o values did not increase between 90 and 120 days post transfer [17].

The results of the peripheral reconstruction model demonstrate that despite a significantly increased reinnervating axonal load, no significant improvement is seen in any of the examined parameters, and that no significant changes in muscle function are seen between 6 and 9 months that cannot be explained simply by muscle growth and physiological changes seen with increasing maturity.

These results suggest that orthotopic and heterotopic muscle grafts undertaken with microneurovascular anastomoses in the setting of the peripheral nervous system are subject to growth in a pattern similar to that of normal (control) muscle and that the transferred muscle continues to mature in a physiological manner[9]. Changes in muscle strength beyond 6 months post transfer are unlikely to be the result of innervation status as no changes are seen in the individually normalized cP_o and wcP_o values, and that these findings concur with previous observations (ie; full reinnervation has occurred by the 6 month time point).

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[7] Significant in the rf group, a trend in the vl group.

[8] Significant in the vl group, a trend in the rf group.

[9] This is surmised from the increase in P_o and wP_o data seen with the transplanted muscles paralleling that seen in the controls.

3.5.2 Facial Reanimation Model

The facial reanimation model employed the rectus femoris muscle transplant coapted to 2 different branches of the facial nerve, with assessment of function again being undertaken at 6 and 9 months post operatively. This experimental model allowed examination of heterotopic muscle transfers in a situation closely simulating that found in clinical facial reanimation. The marginal mandibular branch and ventral ramus of the buccal branch of the facial nerve were chosen as the reinnervating sources for the transfers. These were chosen as they are 2 consistent anatomical structures and have differing axonal loads (the ventral ramus of the buccal branch containing approximately 4 times the axonal load of the marginal mandibular branch).

Few studies have been published examining the functional outcome (in terms of isometric force production) of vascularised functional muscle transfers coapted to cranial nerves. The closest study to the one currently discussed is that of Yamada and Harii [13]. A series of 30 muscle transfers were undertaken with the buccal branch of the facial nerve being used to reinnervate the flaps. Maximal reinnervation of the flaps was found to occur by 5 months with no increases in tetanic force occurring between then and the 12 month time point. Mean cP_o was 8.5%, with muscle mass decreasing to half that at the time of the initial operation (mean wcP_o therefore being 17%).

Frey *et al.* also developed a cranial nerve model for heterotopic muscle grafting, using the scutuloauricularis muscle as the mimic muscle control, and the auricularis rostralis nerve as the innervating source [11]. Rather than examining differing axonal loads, this study looked at the ability of a branch of the facial nerve to reinnervate peripheral muscle (in this case the pectoralis descendens muscle and a piece of the rectus femoris muscle, both approximately twice the mass of the indigenous scutuloauricularis muscle). Orthotopic grafting of the scutuloauricularis muscle results in cP_o of 79% (78% when weight adjusted). Heterotopic grafting of the whole pectoralis muscle results in cP_o of 119% of the control scutuloauricularis muscle, and 88% of the control pectoralis muscle (84% and 114% respectively when weight adjusted). The rectus femoris muscle (for which no same muscle control is available) developed cP_o of 185% of the control

scutuloauricularis muscle, and wcP_o of 103%[10]. The discrepancy between the pectoralis and rectus femoris muscles may lie in the fact that the rectus femoris muscle is a pennate muscle, and that the pieces of rectus femoris weighed on average 20% more than the pectoralis at the time of initial surgery. The cP_o and wcP_o figures for both the grafted scutuloauricularis and pectoralis muscles hint that improved functional recovery[11] (when defined by isometric contractural force) is achievable in the central nervous system setting, when compared to the peripheral nervous system. It is difficult to draw conclusions from the rectus femoris data, beyond stating that larger forces can be generated by the piece of rectus femoris than the indigenous muscle, as there are no same muscle control figures available and again, comparison between pennate and parallel fibred muscles is difficult.

In this current study both the mm group and the bb group used a constant sized muscle reinnervated by differing sized nerves. The results at the 6 month time point indicate superiority of the bb group over the mm group when all values are examined (P_o, wP_o, cP_o, and wcP_o). The bb group developed 50% more force than the mm group (47% when weight adjusted), and when normalized against the control muscles, the bb grafts develop 71% more force (cP_o), 36% when weight adjusted (wcP_o). At the 9 month time point the bb group developed 52% more force than the mm group (79% when weight adjusted), with a 40% greater cP_o value and 68% higher wcP_o. The bb grafts were functionally superior at both time points, generating more force (both raw and control adjusted). It is the weight adjusted figures however that are of greater interest, suggesting that the physiological ability of a given mass of the muscle improves much more in the bb group between the 2 time periods. Comparison of the control muscle data between the 2 points demonstrates that there is no significant increase in the

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[10] These figures are calculated from the mean values given in Table 1 of the paper, though do not agree with values quoted in the text. The control scutuloauricularis muscle P_o and wP_o is (when calculated from the cP_o value given for the rectus femoris muscle in the text) 3.57. This figure does not agree with the value given in Table 1. The pectoralis descendens cP_o and wcP_o values (controlled against the contralateral scutuloauricularis muscle) when calculated from the above value (or those given in Table 1) also fail to agree with those given in the text. The same holds true for the rectus femoris values. The figures calculated and quoted in this text assume P_o and wP_o values of 3.57 for the control scutuloauricularis muscle.

[11] Same muscle control (ie: pectoralis compared to pectoralis).

physiological ability of the muscle that could be attributable to growth and maturation (the difference between this and the findings in the peripheral reconstruction groupings may possibly be explained by the fact that the rabbits were bought in batches, the peripheral experiments being undertaken first, and therefore the animals may have been at different stages of maturation).

Work by Giovanoli *et al.* examined how stepwise increments in the size of transferred muscle pieces coapted to the same recipient branch of the nerve would affect functional outcome [10]. The results were similar to those of Frey *et al.* [11], with cP_o values[12] of 89% for the orthotopic scutuloauricularis muscle graft, 175% for a (tailored) rectus femoris muscle graft double the mass of the scutuloauricularis and 169% for a triple sized piece. When these values were adjusted for weight (wcP_o), they translated as 92.2%, 90.1% and 61.7%. These results demonstrated that the facial nerve could reinnervate a number of muscle fibers larger than that for which it is intended, whilst achieving an improvement in functional outcome. They also demonstrated a distinct limit as to the number of muscle fibers that could be reinnervated – apparent in the data from the failure to improve cP_o values between the double and triple sized pieces of rectus femoris muscle, and the marked decline in wcP_o, suggesting a significant part of the muscle mass (ie: population of denervated muscle fibers) not contributing to the physiological ability of the muscle. These findings echo the results seen in the bb and mm groupings.

In all muscles transferred to the face in this experiment, significant decreases in whole muscle force production were noted (simple comparison of P_o values between the mm, bb and rf groups witnesses this fact). All transfers demonstrated decreased muscle mass, and all had wP_o and wcP_o values below both the control muscles, and those seen in the peripheral transplant groupings. The reason for this is likely to be a reflection of the phenomenon seen in Giovanoli's work, work by Cederna *et al.* and van der Meulen *et al.* [10] [21] [22] namely, the nerves used to reinnervate the muscle simply do not have the ability to reinnervate all the muscle fibers contained within the transplant (otherwise it would be expected from the studies detailed above that 80% - 90% cP_o could be achieved[10] [11]). The difference noted between the mm and bb groups is also

[12] The control for all grafted muscles in the study was the contra lateral scutuloauricularis.

likely to be a reflection of the above phenomenon, with the physiological superiority of the bb group likely being derived from the increased axonal numbers.

The change in physiological performance of the bb group between 6 and 9 months (and the relative lack of change seen in the mm group) is also possibly related to axonal load in the reinnervating nerve. In the peripheral setting completion of the reinnervation process is seen at 6 months, as demonstrated by the data from this study and that in others discussed above. This is probably due to the fact that the indigenous nerve has the ability to reinnervate the orthotopically placed muscle fully[13], as increases in motor unit size appears to compensate for any neuronal loss across the neurorrhaphy and do so without reaching the point where the motor unit size is so large that physiologic performance starts to decrease (ie: there appears to be built in redundancy within the nerve to compensate for injury without loss of performance). Such "full" reinnervation is undertaken by the myelinated axons that have traversed the neurorrhaphy by the 6 month time point. In the case of the mm and bb groups the number of myelinated axons reaching the muscle fibers at 6 months is insufficient to fully reinnervate them (though there are more axons present in the bb group and therefore these muscles have improved physiological performance). It would seem however that a significant number of axons continue to reinnervate muscle fibres in the bb group up until the 9 month time point. The significant difference between the mm and bb groups at the later time points is likely to be due to the larger population of maturing axons seen in the latter group. The fact that no significant increase in wcP_o is seen in the mm group between the 2 time points may be due to the fact that the functional capacity of the marginal mandibular nerve has been reached by the 6 month time point and there are not sufficient axons within the nerve to bring about any further improvement in performance. The ventral ramus of the buccal branch of the facial nerve however has sufficient numbers of axons continuing to mature that further increases in physiological performance are seen between the 6 and 9 month time points.

[13] In this case "fully" is taken as the maximal level of reinnervation (determined by physiological performance) that can be attained in a functional muscle transfer.

3.5.3 Discussion – Force Frequency Curves

The arrival of an action potential at a motor end plate causes release of acetylcholine which acts on receptors on the sarcolemma of the muscle fiber, inducing an action potential which rapidly spreads, via the T – system throughout the whole muscle cell. This action potential causes release of Ca^{2+} from the closely related sarcoplasmic reticulum, resulting in a rise in the intracellular Ca^{2+} concentration. This initiates the actin-myosin binding system, and the sliding cycle of the actin-myosin complex is completed with a resultant shortening in sarcomere length. Calcium ions are rapidly removed from the intra cellular space by active transport mechanisms, the actin-myosin complex dissociates and the muscle fiber relaxes[14].

Single stimuli invariably result in maximal Ca^{2+} release producing a maximal single twitch of a striated muscle fiber. Single stimulus does not result in maximal muscle shortening as it is too brief to maintain the relatively slow filament sliding to its end position. Only when a second stimulus arrives during the first twitch does further shortening occur. Repeated stimuli result in the superposition of single twitches, mechanical summation. If the frequency of stimuli is increased so that intervals between action potentials becomes less than 1/3 of the time required for a single twitch the twitches fuse and the maximal possible contraction of the muscle fibre/motor unit occurs [23].

The frequency of stimuli required to produce maximal force is also known as the fusion frequency and differs between muscles depending on fiber type composition. Predominantly slow muscles exhibit slower tension rises within the muscle coupled with a longer relaxation phase. Fast muscles demonstrate much quicker times to peek tension together with rapid relaxation. Fusion frequencies for slow muscles are therefore lower than those for fast muscles, as the total fiber activation time is longer, presenting greater opportunity for interruption of the preceding twitch. Typically slow mammalian muscle fuses at around 20Hz, whilst fast muscles have fusion frequencies of up to 60 – 100Hz [23] [24]. In muscle recovering from denervation both the rise and relaxation

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[14] This is a simplified description of the events involved in muscle contraction, for a fuller account please see physiology texts such as that by Despopoulos. Despopoulos, A. and S. Silbernagl, *Colour Atlas of Physiology.* 1991, New York: Thieme Medical PublishersLtd..

phases of twitch response are known to be prolonged and the fusion frequency is lower (the latter being a function of the former) [25].

Although several authors describe in their methods the use of incremental stimulation frequencies in their experiments, only a few document their results in respect to this aspect of their work. From his extensive experience with the rectus femoris muscle in the rabbit Frey estimates that the fusion frequency for the rectus femoris muscle is approximately 50Hz in its natural state [26]. This correlates well with the findings from this study where the control flaps reached fusion frequency at approximately 50Hz in all groups, and the work by Yamada and Harii who also estimated fusion frequency to occur at around 50Hz in their control flaps [13]. In their series of canine gracilis flaps Das *et al.* did not note any variance in fusion frequency between operated and control flaps, with complete recovery of wcP_o values suggesting complete reinnervation of all fibers contained within the transfer[15]. Although Guelinckx *et al.* also reported on fusion frequency/force relationships in their series of rectus femoris orthotopic transfers in the rabbit, complete fusion had yet to occur even at frequencies of 120Hz. This data contradicts previous findings for this particular muscle and general observations on mammalian muscles as a whole [23] [24] [26] [11] [9] [10].

In this series the operated muscles all reached tetany at lower frequencies than the control muscles. The peripheral muscle groupings demonstrated fusion frequencies of approximately 40Hz at both time points, whilst in the facial reanimation model groups, the mm flaps required 20Hz stimulation to produce maximal force compared to 30Hz seen with the bb transplants. The reasons for this down shift in the stimulation frequency required to produce tetany is likely to be a combination of a residual population of denervated fibers altering twitch contraction properties [25] (in the case of the peripheral groupings where no changes or a decrease in type I fibers have been previously reported following orthotopic grafting [9] [19]), and an increase in the type I fiber population in the case of the facial reanimation model groupings (as has been found in

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[15] Somewhat confusingly the percentage composition of the transfers by slow twitch fibres increased to 48% compared to 20% in the controls, which one might assume would predispose the transplants to lower fusion frequencies than those of the control muscles, but in this case failed to do so.

previous peripheral muscle transplants coapted to the facial nerve) [10] [11] [13]. The lower fusion frequency seen in the mm group compared to the bb group may be a reflection of an increased proportion of denervated muscle fibers present in these grafts.

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Chapter 4

Results

Nerve Histomorphometry

4.1 Introduction

Division and repair of the motor nerve to a muscle frequently results in a decreased level of function compared to premorbid status [1] [2]. Although some authors have reported full or near full recovery of skeletal muscle force production following such procedures [3] [4], care must be taken in the interpretation of such results as the "biology of nerve regeneration is highly species specific" [5]. Studies that provide rapid reinnervation of small muscles by relatively large numbers of regenerating fibres report near full recovery of function, though those involving reinnervation of larger muscles with fewer axons generally report force deficits [1] [6] [7] [8] [9].

Muscle atrophy plays a role in the decreased force production with a reduction in overall muscle mass following nerve repair [10] [1], though when normalized for muscle fibre cross sectional area (controlling for muscle fibre atrophy) a specific force deficit still exists in the muscle. Experimental evidence points to residual populations of denervated muscle fibres being responsible for the decreased force production [1] [11] [24]. Neural escape at the site of neurorrhaphy leading to a decreased reinnervating motor neuron population has been postulated as a likely cause of this [12] [13] [14] [15] [16] [17].

The nature of functional muscle transfer requires the division of the donor muscle's motor nerve, with coaptation at the recipient site to a foreign motor input. This makes the transfer vulnerable to the same problems faced by any muscle that has its motor nerve supply interrupted, though it is subject to the additional problem that the reinnervating neural supply is not that of its native motor branch, and that there is likely to be a mismatch in terms of size between the two. It is known that axonal size (diameter) governs how many muscle fibres a given axon can reinnervate [18] [19] [20], therefore increasing the size of the motor neural input (in terms of total axonal cross sectional area) is likely to increase the population of transferred muscle fibres that are reinnervated, thereby improving functional outcome of the muscle transplant.

A reduction in the number of residual denervated fibres in peripheral limb reconstruction muscle transfers (the most likely factor responsible for the relatively poor performance of these grafts) may possibly provide the key to improving outcomes in this field. Likewise functional muscle transfers undertaken for facial reanimation surgery, although providing

an excellent result in the majority of cases, also suffer from unpredictability. Minimal movement and over activity are both seen post transfer, with the overall results best described as a spectrum. The proposed theory in this work is that variation of neural input into the transferred flaps is responsible for the differing outcomes seen. Little work has examined the reinnervation characteristics of central nerves upon facial muscles or vascularised muscle transfers, though it is likely that muscles transferred to the face and coapted to the facial nerve are subject to the same phenomena as those described above for the peripheral nervous system. If this is true therefore, by varying the VII motor input to a muscle transfer the degree of function (movement) achieved post transfer could be varied according to need, allowing tailoring of the operative result and avoiding under or over activity of the muscle.

4.2 Aims

To see if differing sized axonal loads derived from the facial nerve produce differing axonal counts and total axonal cross sectional areas in the motor branch to the rectus femoris muscle distal to the site of neurorrhaphy: Thus examining cranial nerve reinnervation and re-growth characteristics.

To see if increasing both the axonal count and axonal total cross sectional area of the motor input reinnervating the rectus femoris results in an improved motor neural profile distal to the neurorrhaphy site, when compared to its native branch (ie; in a peripheral nerve setting): Thus examining peripheral nerve reinnervation characteristics.

To assess if these findings correlate with physiological outcomes observed in these models.

4.3 Methods

Forty eight male 3.0 – 3.5 kg New Zealand White rabbits were divided in 4 groups as detailed previously. The rectus femoris muscle was used as a vascularised functional muscle transfer placed either orthotopically (coapted to its native nerve for its source of reinnervation) or heterotopically (coapted to the larger neighboring motor nerve to vastus lateralis) in the leg, to simulate the peripheral limb reconstruction scenario. The muscle was also transferred to the face where it was coapted to either the marginal mandibular branch or the larger ventral ramus of the buccal branch of the facial nerve to simulate the clinical facial reanimation setting.

Following physiological assessment the motor nerve to the muscle was dissected out, divided at the site of neurorrhaphy into proximal and distal segments and processed for semi thin sectioning as described in Chapter 2 section 2.3. These sections were examined using light microscopy, with the nerve sections being captured field by field using a digital camera linked to a microscope and montaged to produce an overall image of the section. This was then printed out using a large format printer at x2250 magnification with all axons contained within the section being counted and sized using a semi automated, computer assisted technique as detailed in Chapter 2 section 2.3.3.

4.4 Results

4.4.1 Peripheral Reconstruction Model

The motor nerve to the rectus femoris muscle was divided into a proximal and distal segment at the site of neurorrhaphy. The proximal segment therefore represented the composition of the "donor" nerve used reinnervate the muscle transfer, the distal segment the neural component actually reinnervating the flap. The donor nerves used in this experiment were the motor nerve to rectus femoris (rf) and the larger motor nerve to vastus lateralis (vl).

Comparison of the proximal segments of the motor nerves was undertaken to establish whether the motor nerve to vastus lateralis was indeed significantly larger than that of the rectus femoris nerve. The total axonal count (ac) in the "donor" nerves revealed a significant difference between the rf and vl nerves at the 6 month time point $p < 0.001$ (6rf ac 1649.2 [stdev 158.48, SEM 70.87] vs. 6vl ac 3231.4 [stdev 225.18, SEM 91.93]). A similar finding was noted in the axon counts of the donor nerves at the 9 month time point, $p = 0.002$ (9rf ac 1588.4 [stdev 463.29, SEM 189.14] vs. 9vl ac 3164 [stdev 748.61, SEM 305.62]), analyzed using Mann Whitney rank sum test due to data failing equal variance test). This data is represented graphically in figure 4.4.1.

The total axonal cross sectional area (aa) was also examined to confirm that there was a significant difference between the rf and vl nerves. The mean total axonal cross sectional area for the 6rf group was 0.053 mm^2 (stdev 0.013, SEM 0.0059), the 6vl group mean being 0.071 mm^2 (stdev 0.013, SEM 0.0053). The difference between these was significant $p = 0.042$. The values for the 9 month groupings were 0.048 mm^2 (stdev 0.014, SEM 0.0058) and 0.069 mm^2 (stdev 0.013, SEM 0.0053) respectively, again the difference was significant $p = 0.027$. This data is represented graphically in figure 4.4.2.

Comparison of the distal nerve segments for the aforementioned parameters was then undertaken to assess if any differences existed in the axonal size and number. No differences were noted in the axonal counts in the distal nerve segments between the rf and vl groups at either the 6 month ($p = 0.62$) or 9 month ($p = 0.83$) time points. The axonal counts for the groupings were 6rf ac 1669.2 (stdev 345.55, SEM 154.53) vs. 6vl

ac 1769.5 (stdev 304.63, SEM 124.36) and 9rf ac 1826 (stdev 402.56, SEM 180.03) vs. 9vl ac 1906.8 (stdev 739.19, SEM 301.77). This data is represented graphically in figure 4.4.3.

Total axonal cross sectional area in the distal segments demonstrated no significant difference between the 6rf and 6vl groups ($p = 0.32$) (6rf aa 0.028 mm^2 [stdev 0.004, SEM 0.0019] vs. 6vl aa 0.024 mm^2 [stdev 0.008, SEM 0.0034]). A significant difference was noted however between the rf and vl groupings at the 9 month time point ($p = 0.044$) (9rf aa 0.039 mm^2 [stdev 0.011, SEM 0.0053] vs. 9vl aa 0.024 mm^2 [stdev 0.008, SEM 0.0034]). The data for this is represented graphically in figure 4.4.4. No significant differences were noted between time points in either the rf or vl groupings for either ac or aa parameters.

The percentage of axons crossing the neurorrhaphy was examined. At the 6 month time point 93.2% (stdev 21.58, SEM 10.79) of axons crossed the nerve repair site in the rf group compared to 65.2% (stdev 27.59, SEM 10.43) in the vl group, this was not significant $p = 0.11$. At the 9 month time point 124.9% (stdev 55.10, SEM 24.6) of the number of axons found in the proximal segment were present in the distal nerve segment in the rf group, compared to 57.07% (stdev 20.75, SEM 9.28) in the vl group ($p = 0.016$, analyzed using the Man Whitney rank sum test due to failure of data normality). This difference was significant. The above data is represented graphically in figure 4.4.5.

The percentage of the total axonal area seen in the distal segment compared to the proximal one was also examined. At the 6 month time point 59.2% (stdev 27.28, SEM 13.64) of the proximal axonal area was found in the distal segment of the rf group, compared to 38.2% (stdev 13.89, SEM 5.67) in the vl group, $p = 0.14$. At the 9 month time point these values were found to be 83.34% (stdev 40.23, SEM 17.99) vs. 37.63 (stdev 15.59, SEM 7.13) respectively, this difference being significant $p = 0.046$. This data is represented graphically in figure 4.4.6

Figure 4.4.1: Proximal Axonal Counts in Peripheral Reconstruction Model. The groups are labeled on the x-axis, the mean axonal count on the y-axis. The mean value for each group is given within the column, the SEM value is detailed above the respective error bar. Significant values are denoted by an overhead line between the respective groups.

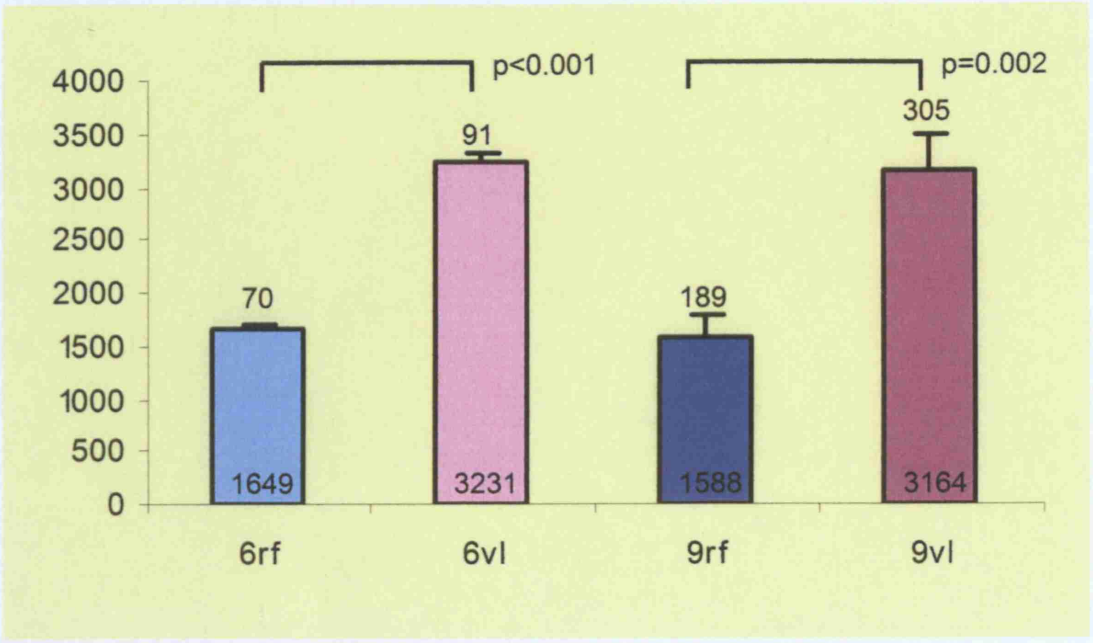


Figure 4.4.2: Proximal Axonal Area in Peripheral Reconstruction Model. The groups are labeled on the x-axis, the axonal area (mm^2) on the y-axis. The mean value for each group is given within the column, the SEM value is detailed above the respective error bar. Significant values are denoted by an overhead line between the respective groups.

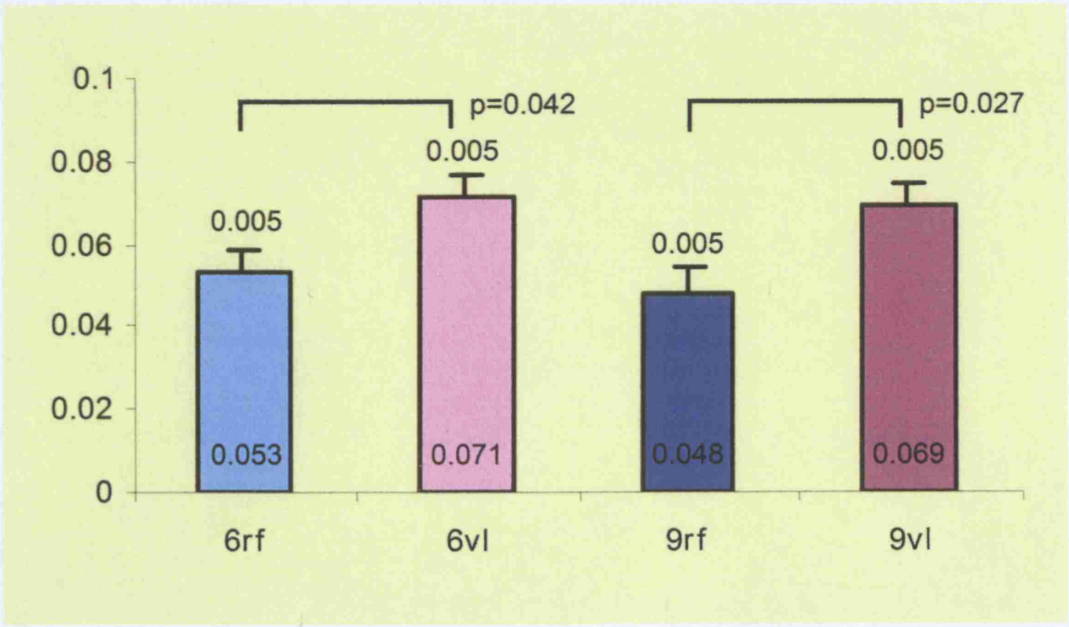


Figure 4.4.3: Distal Axonal Counts in Peripheral Reconstruction Model. The groups are labeled on the x-axis, the mean axonal count on the y-axis. The mean value for each group is given within the column, the SEM value is detailed above the respective error bar. Significant values are denoted by an overhead line between the respective groups.

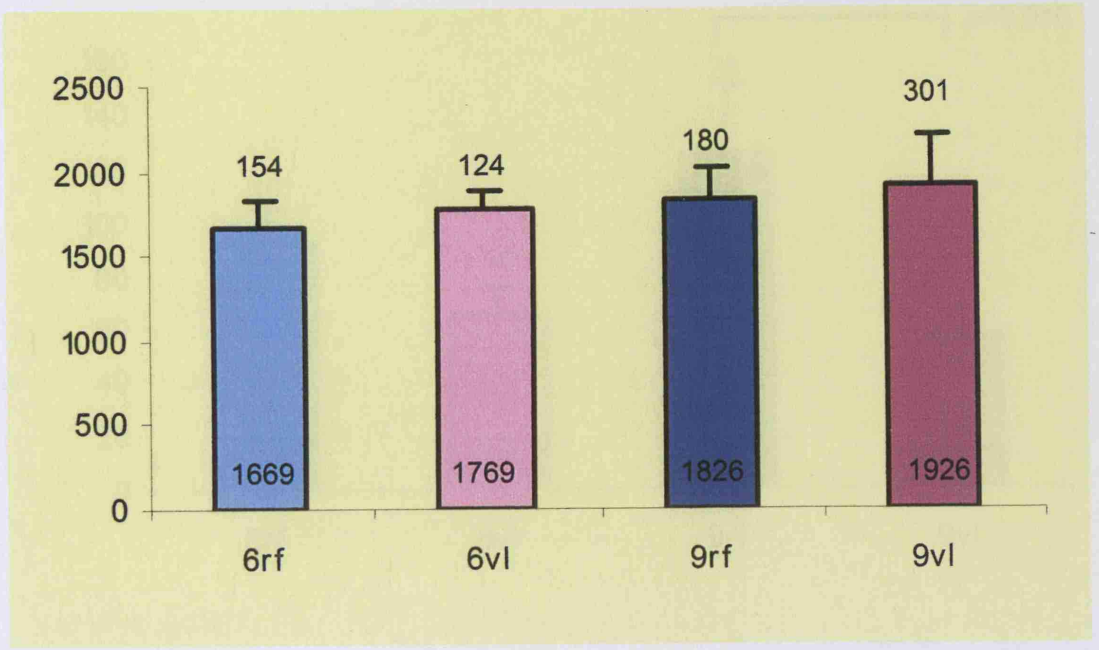


Figure 4.4.4: Distal Axonal Area in Peripheral Reconstruction Model. The groups are labeled on the x-axis, the axonal area (mm^2) on the y-axis. The mean value for each group is given within the column, the SEM value is detailed above the respective error bar. Significant values are denoted by an overhead line between the respective groups.

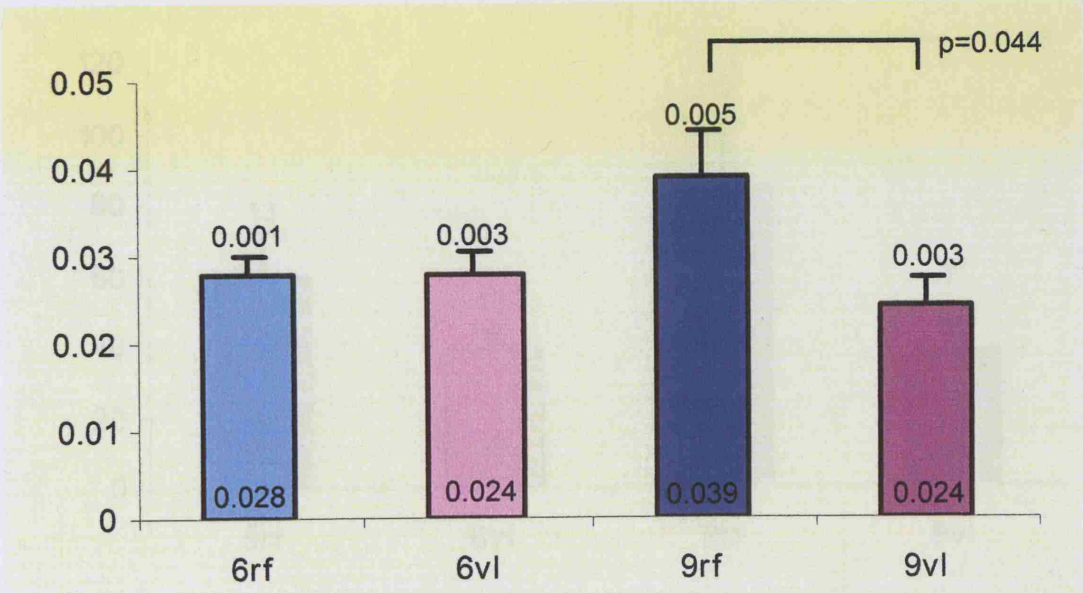


Figure 4.4.5: Percentage of axons crossing the neurrorhaphy. The groups are labeled on the x-axis, the mean percentages on the y-axis. The mean value for each group is given within the column, the SEM value is detailed above the respective error bar. Significant values are denoted by an overhead line between the respective groups.

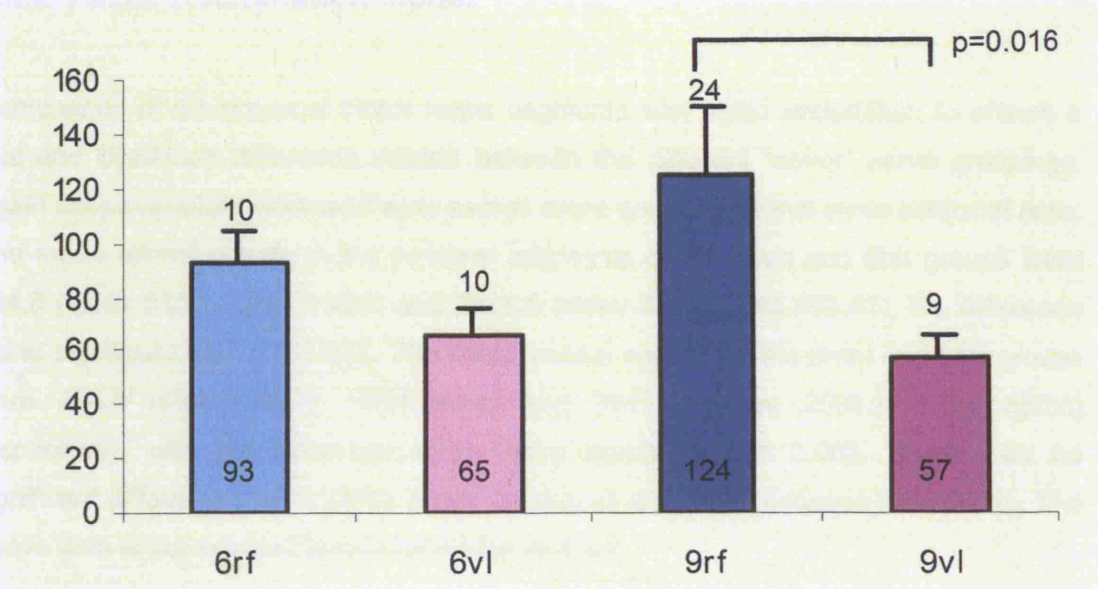
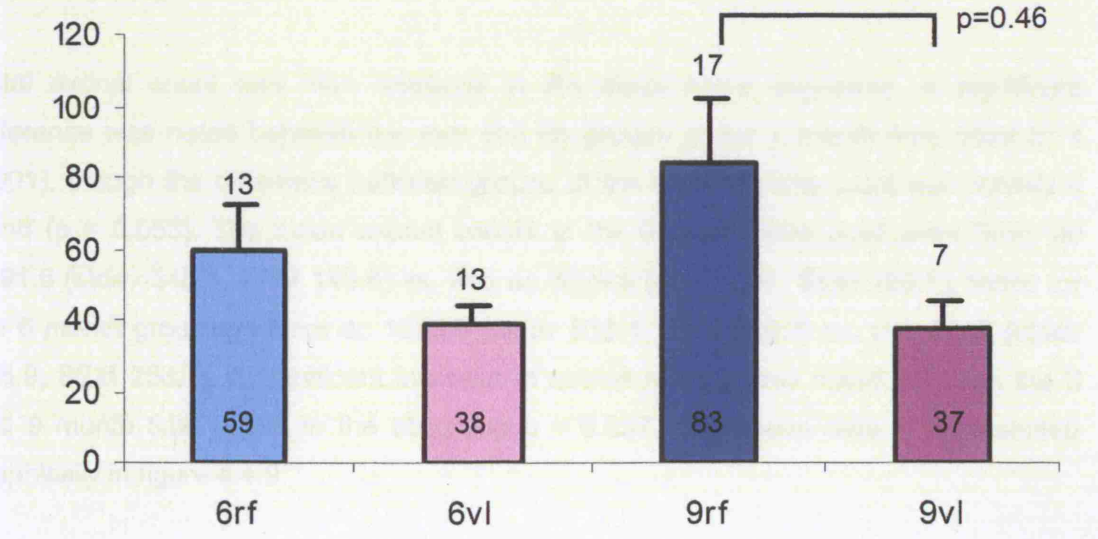


Figure 4.4.6: Axonal cross sectional area crossing the neurrorhaphy. The groups are labeled on the x-axis, the mean percentages on the y-axis. The mean value for each group is given within the column, the SEM value is detailed above the respective error bar. Significant values are denoted by an overhead line between the respective groups.



4.4.2 Facial Reanimation Model

Comparison of the proximal motor nerve segments was again undertaken to ensure a real and significant difference existed between the different "donor" nerve groupings. Again the parameters assessed were axonal count and total axonal cross sectional area. The mean axonal counts in the proximal segments of the 6mm and 6bb groups were 634.8 (stdev 335.3, SEM 136.8) and 2626.5 (stdev 1224, SEM 499.83), the difference being significant with $p = 0.003$. The mean axonal counts for the 9mm and 9bb groups were 730.8 (stdev 202.1, SEM 82.5) and 3911.8 (stdev 2098.3, SEM 856.8) respectively, with this difference again being significant $p = 0.002$. There were no significant differences seen within either the mm or bb groups between time points. The above data is represented graphically in figure 4.4.7.

Analysis of total axonal cross sectional area demonstrated significant differences between groups at both the 6 month ($p = 0.009$, assessed using Mann Whitney rank sum test due failure of equal variance test) and 9 month time points ($p = 0.004$). The mean area values were as follows: 6mm aa 0.012 mm^2 (stdev 0.004, SEM 0.001) vs. 6bb aa 0.045 mm^2 (stdev 0.02, SEM 0.008). 9mm aa 0.015 mm^2 (stdev 0.015, SEM 0.006) vs. 9bb aa 0.040 mm^2 (stdev 0.005, SEM 0.002). The above data is represented graphically in figure 4.4.8.

Total axonal count was then analyzed in the distal nerve segments. A significant difference was noted between the mm and bb groups at the 9 month time point ($p < 0.001$), though the difference between groups at the 6 month time point was merely a trend ($p = 0.053$). The mean axonal counts at the 9 month time point were 9mm ac 1491.6 (stdev 345.1, SEM 140.8) vs. 9bb ac 3029.5 (stdev 637, SEM 260.1), those for the 6 month groupings 6mm ac 1352.6 (stdev 538.4, SEM 240.7) vs. 6bb 2147 (stdev 628.9, SEM 256.7). A significant increase in axonal number was noted between the 6 and 9 month time points in the bb group $p = 0.037$. The above data is represented graphically in figure 4.4.9.

Total axonal cross sectional area demonstrated significant differences between mm and bb groupings at both time points (6 months $p = 0.002$, 9 months $p = 0.002$). The mean axonal cross sectional area in the distal nerve segments were 0.006 mm^2 (stdev 0.009,

SEM 0.004) vs. 0.025 mm^2 (stdev 0.001, SEM 0.0006) for the 6mm and 6bb groups respectively, and 0.014 mm^2 (stdev 0.004, SEM 0.001) vs. 0.036 mm^2 (stdev 0.012, SEM 0.005) for the 9mm and 9bb groups. A significant increase was noted between time points in the mm groupings ($p = 0.004$, analyzed using Mann Whitney rank sum test due to failure of equal variance). The above data is represented graphically in figure 4.4.10.

The percentage of axons found in the distal nerve segment compared to that found in the proximal segment was then examined. At the 6 month time point 264.6% (stdev 123.1, SEM 55.05) of the number of axons in the proximal segment were noted in the distal segment in the mm group, compared to 102.7% (stdev 55.31, SEM 22.58) in the bb group ($p = 0.017$). At the 9 month time point these values were 214.6% (stdev 38.3, SEM 15.6) vs. 94.9% (stdev 49.52, 20.21) for the mm and bb groups respectively ($p < 0.001$). The above data is represented graphically in figure 4.4.11.

No difference between the groups was noted at either time point when the mean total axonal cross sectional areas in the distal segment were compared to those in the proximal segments. The percentage values were 64.44% (stdev 18.59, SEM 8.31) vs. 66.19% (stdev 7.81, SEM 3.19) for the 6mm and 6bb groups respectively $p = 0.62$, and 87.35% (stdev 17.63, SEM 7.19) vs. 81.33% (stdev 14.24, SEM 5.81) for the 9mm and 9b groups ($p = 0.62$). The above data is represented graphically in figure 4.4.12.

4.5 Discussion

As stated previously in the text the recovery of muscle function following peripheral nerve injury has been persistently documented as poor (excepting in certain isolated circumstances), or to quote one author "the results of peripheral nerve repair in the three decades following World War II continue to demonstrate lack of excellent recovery of function" [21]. The idea of improving outcome by increasing the levels of motor fibres reinnervating a muscle has been identified by previous authors though conflicting results have been reported [21] [10]. It is known that the number of muscle fibres that can potentially be reinnervated by a single motor axon is proportional to the diameter of the axon [18] [20], and that there is considerable ability of the reinnervating axons to increase the size of their motor unit territory to encompass nearby denervated muscle

Figure 4.4.7: Proximal Axonal Counts in Facial Reanimation Model. The groups are labeled on the x-axis, the mean axonal count on the y-axis. The mean value for each group is given within the column, the SEM value is detailed above the respective error bar. Significant values are denoted by an overhead line between the respective groups.

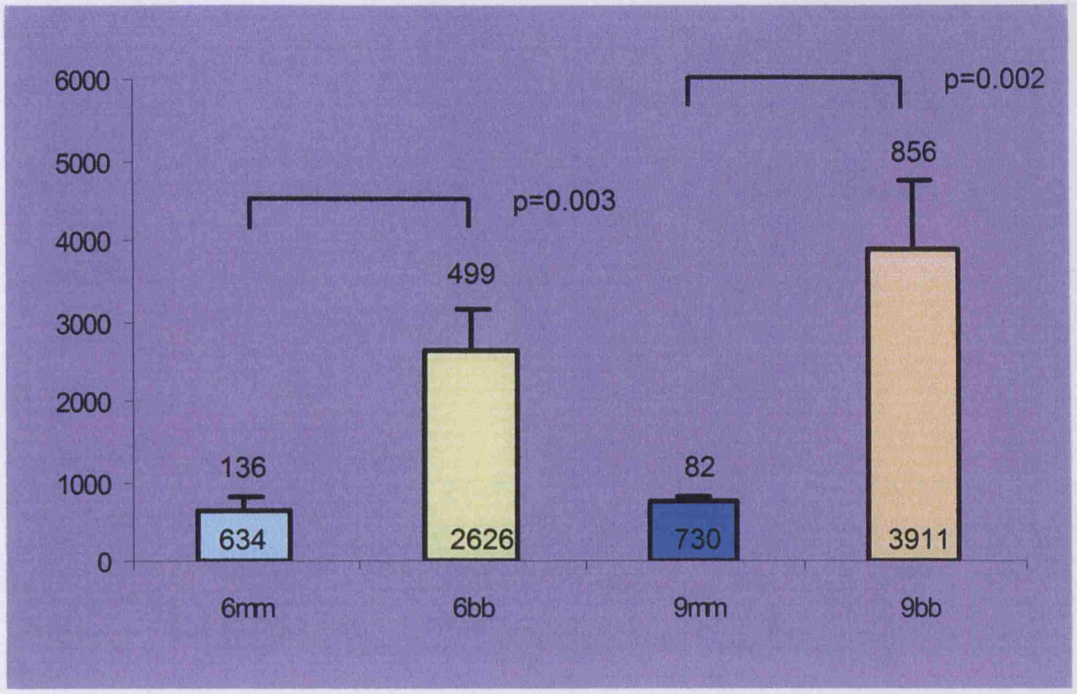


Figure 4.4.8: Proximal Axonal Areas in Facial Reanimation Model. The groups are labeled on the x-axis, the axonal area (mm^2) on the y-axis. The mean value for each group is given within the column, the SEM value is detailed above the respective error bar. Significant values are denoted by an overhead line between the respective groups.

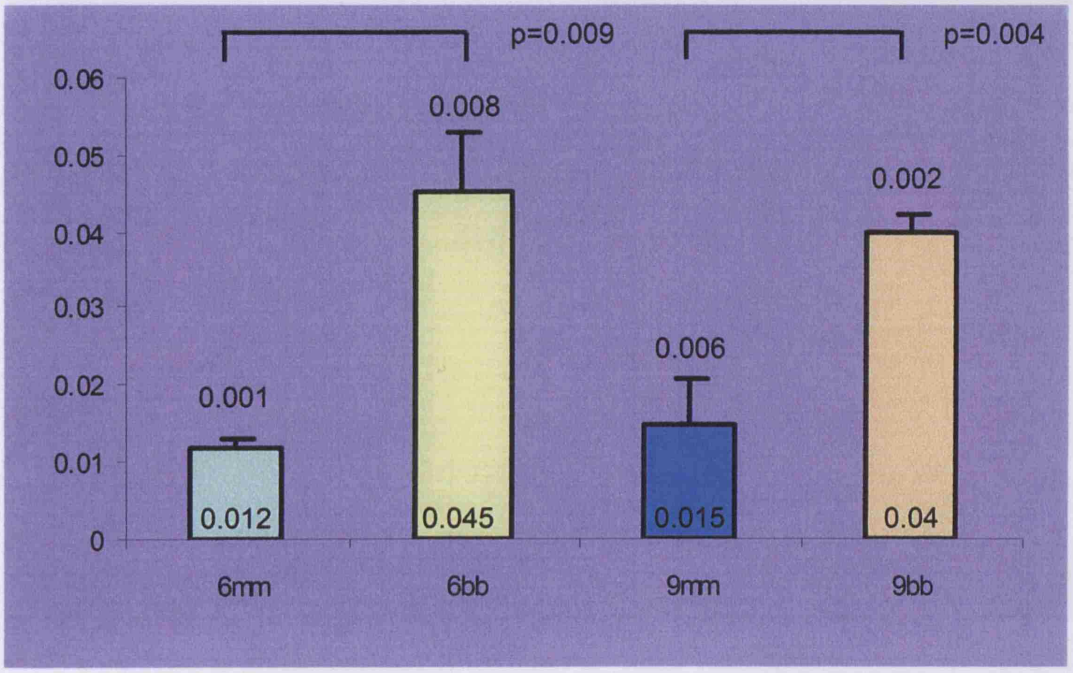


Figure 4.4.9: Distal Axonal Counts in Facial Reanimation Model. The groups are labeled on the x-axis, the mean axonal count on the y-axis. The mean value for each group is given within the column, the SEM value is detailed above the respective error bar. Significant values are denoted by an overhead line between the respective groups.

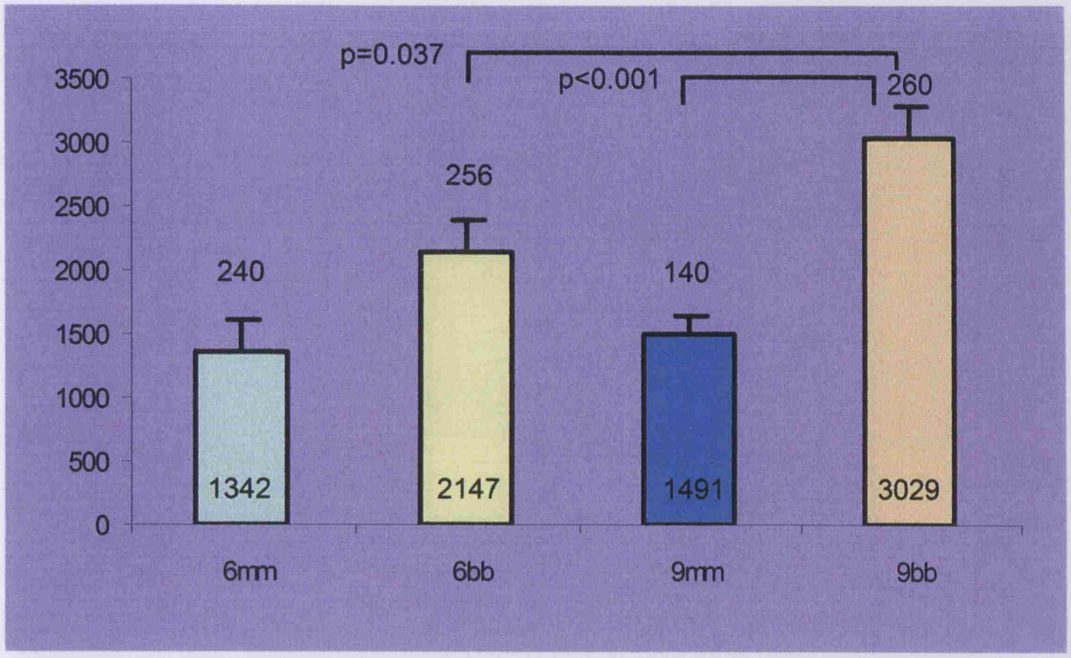


Figure 4.4.10: Distal Axonal Areas in Facial Reanimation Model. The groups are labeled on the x-axis, the axonal area (mm^2) on the y-axis. The mean value for each group is given within the column, the SEM value is detailed above the respective error bar. Significant values are denoted by an overhead line between the respective groups.

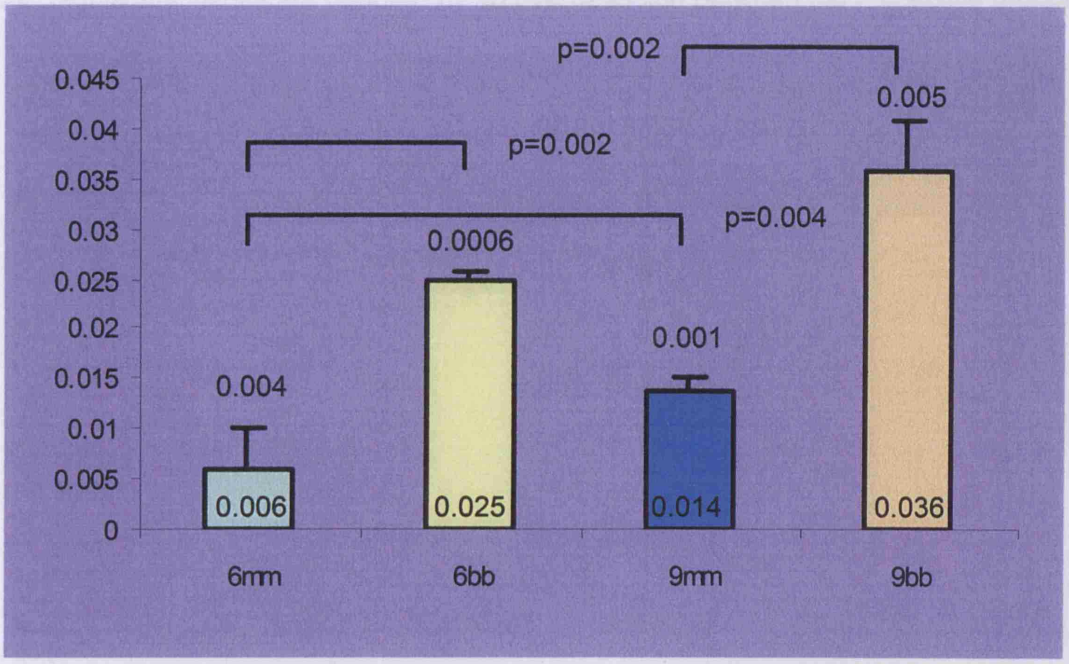


Figure 4.4.11: Percentage of axons crossing the neurrorhaphy. The groups are labeled on the x-axis, the mean percentages on the y-axis. The mean value for each group is given within the column, the SEM value is detailed above the respective error bar. Significant values are denoted by an overhead line between the respective groups.

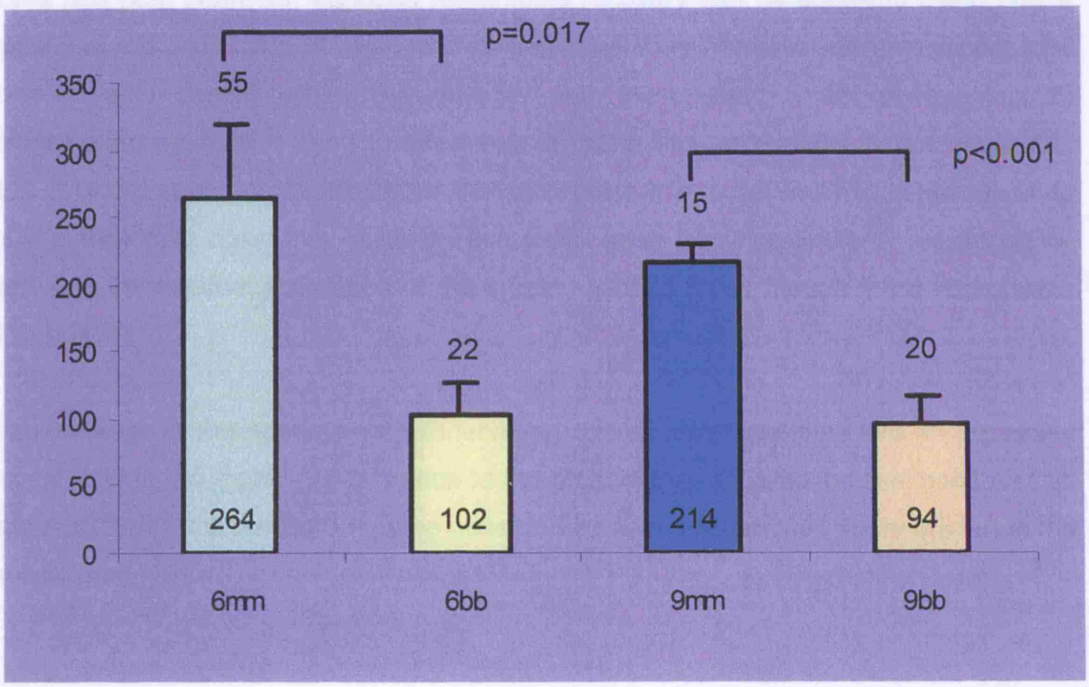
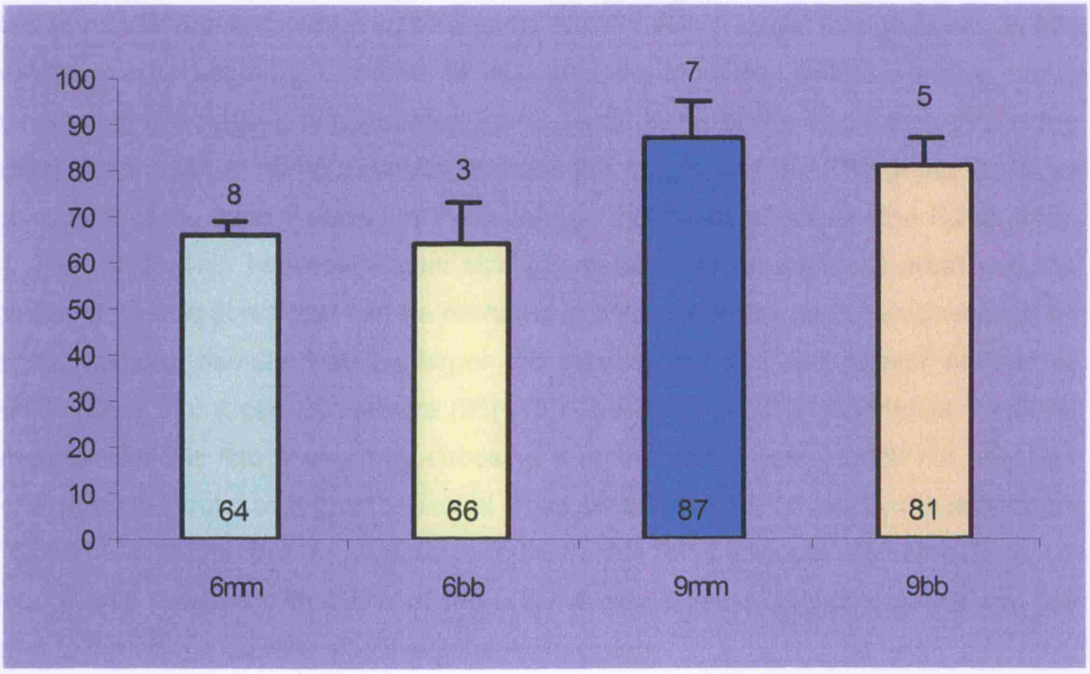


Figure 4.4.12: Axonal cross sectional area crossing the neurrorhaphy. The groups are labeled on the x-axis, the mean percentages on the y-axis. The mean value for each group is given within the column, the SEM value is detailed above the respective error bar. Significant values are denoted by an overhead line between the respective groups.



fibres [22] [23]. However, although remarkable recovery can be achieved using only a fraction of the population of reinnervating motor neurons normally seen significant total force defects persist within the muscle, and even when a full complement of reinnervating neurons is used to effect reinnervation this force deficit is not eradicated [11]. It would seem logical therefore that increasing the motor input to a reinnervating muscle may over come this residual force deficit seen following axotomy and repair by capturing the residual population of denervated muscle fibres though to be responsible [11] [1] [24].

If an increase in the proximal or reinnervating axonal load translates into an increased axonal load in the motor nerve branch to the muscle then there is the likelihood that an improved functional outcome may be observed as fewer denervated fibers are left in the muscle graft [10].

4.5.1 Peripheral Reconstruction Model

As previous authors have postulated an increase in the number of reinnervating axons would improve outcomes when considering muscle reinnervation [10] [23] [21], the paradigm for this experiment was designed so as to compare the native motor nerve used to reinnervate a constant sized muscle transfer with a larger foreign nerve. In this way the model used by Cederna *et al.* could be modified, with the native nerve representing the R100 group (repaired nerve, with 100% of the nerve included in the repair) and the larger nerve to vastus lateralis the corollary of the R50 group (repaired nerve, 50% of the axon included in the repair), in this cases effectively the R200 group [1]. The relationship between axonal size (diameter or cross sectional area) and the number of muscle fibers that can be recruited into its motor unit has been described by several authors, namely that the larger the axonal diameter, the greater number of muscle fibers that it can reinnervate [20] [18]. The design of the experiment therefore aimed to take this into account by choosing a reinnervating nerve which not only had more axons in it but also a greater axonal cross sectional area. To modify the annotation of Cederna *et al.* the native motor nerve to the rectus femoris would represent the A100 group (nerve repaired with 100% of the original axonal cross sectional area), with the nerve to the vastus lateralis effectively the A140 group.

The differences in total proximal axonal count and axonal cross sectional area between the rf and vl groups were significant at both the 6 and 9 month time points. The motor nerve to vastus lateralis contained on average 202% of the axons of that of the rectus femoris nerve, with on average 138% of the total axonal cross sectional area. This demonstrates that as a model for increased axonal loading to reinnervate a muscle flap the motor nerve to vastus lateralis fulfilled similar characteristics to models used by other authors examining the effects of overloading or hyper reinnervation[1].

Six months following the operative procedures the axonal counts in the nerve segment distal to the neurorrhaphy were almost identical in both groups, the rf group having a mean count of 1649.2 axons, compared to 1769.5 in the vl group. Axonal cross sectional area also demonstrated no significant difference between the groups at this time point with the rf group having a total of 0.028 mm^2 compared to 0.024 mm^2 in the vl group. These values equated to 93% of the rf axons crossing the neurorrhaphy compared to 65% of the vl group (not significant) and these represented 59% of the pre-neurorrhaphy axonal area in the rf group and 38% in the vl group (again the differences between the groups not being significant).

At the 9 month time point axonal counts distal to the neurorrhaphy were again almost identical with 1826 in the rf group, compared to 1906.8 in the vl group. The axonal area did however demonstrate a significant difference between the 2 groups with the rf group having an increased neural area compared to the vl group (0.039 mm^2 compared to 0.024 mm^2). Equivalent percentages of the proximal values were therefore 117.5% of the proximal axon count and 83.3% of the proximal axonal area in the rf group, with

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[1] MacKinnon *et al.* used overloading axonal number ratios of 2.5:1 and 3.5:1 when examining the subject, whilst Kuiken *et al.* examined the effects of increasing the axonal input count by 2.6, 3.0, 5.4 and 12 times . Mackinnon, S.E., et al., *Selection of optimal axon ratio for nerve regeneration.* Ann Plast Surg, 1989. **23**: p. 129-134. . Kuiken, T.A., D.S. Childress, and W.Z. Rymer, *The hyper-reinnervation of rat skeletal muscle.* Brain Research, 1995. **676**: p. 113.. Taxt investigated the effects of increasing the reinnervating motor axon number by a factor of 2 in his work on the subject . Taxt, T., *Motor unit numbres, motor unit sizes and innervation of single muscle fibers in hyperinnervated adult mouse soleus muscle.* Acta Physiol Scand, 1983. **117**: p. 571-580..

50.5% and 37.6% respectively for the vl group. These percentage changes between the groups were also significant.

The above data implies that despite improved odds in getting axons to traverse a neurorrhaphy (by increasing both absolute numbers and total axonal size or area) in this experiment, there was a failure of the distal nerve to display any increase in either number or total area of axons when an over sized nerve was coapted to it. The percentage of axons crossing the repair site both in terms of number and overall area was far superior in the rf group suggesting that the failure of improved axonal growth across the repair was unlikely to be due to a physical barrier such as scar tissue (since presumably both repairs would be subject to similar tissue conditions).

These results concur with the findings of the physiological assessments carried out on the muscle transfers, where no differences were found in functional outcome between the groups; A similar number of axons are found in the motor nerve in both groups at both time points, with no differences between either group (or inter-group) in terms of total axonal area excepting between the rf and vl groups at the 9 month time point. Although an improved neuronal profile was seen in the 9rf group nerve morphology studies, this failed to improve outcome in terms of function at 9 months post transfer, though there is the possibility that further improvements in function may have become apparent given the further passage of time.

Adequate reinnervation of a muscle requires the directional in-growth of motor neurons down the distal nerve sheath, thus being appropriately directed towards the site of previous motor end plates and muscle fibres [25] [26] [27]. This pattern of regrowth allows an increase in motor unit size within the reinnervated muscle to accommodate for decreases in neural input and thus minimize the denervated fibre population [20]. Direct end to end nerve suture promotes this type of nerve regrowth (compared to techniques such as nerve – muscle implantation) and immediate repair of the divided nerve provides the optimum conditions for this to occur in.

Previous authors have examined outcomes following overloading of the neural input at the time of repair following nerve division. MacKinnon *et al.* used the rat model to investigate the effects of increasing the neural input to the peroneal nerve, utilizing the

posterior tibial or sciatic nerves as the donor units to gain a ratio advantage of 2.5:1 and 3.5:1 respectively. The results of this study demonstrated extremely poor outcomes in both of the over sized groups whilst showing good recovery of function in the indigenous nerve repair group (1:1 ratio), prompting the authors to suggest that appropriateness rather than number of axons results in optimal reinnervation [21]. The reinnervation of adult mouse soleus muscle by double the normal number of motor axons from 2 foreign nerves was examined by Taxt [23]. This study found that all the motor axons used to reinnervate the muscle formed functioning motor units, though a decrease in absolute motor unit size was noted (due to the lack of denervated muscle fibres for the increased population of motor neurons to make synapses with). Overall approximately double the number of motor units were formed in the muscles, with approximately 20 -30% of the muscle fibers receiving poly neuronal innervation. These findings suggest that increases in axonal number may indeed form useful and permanent synapses when used to reinnervate a muscle, however no functional assessment of the muscle was undertaken as part of this study. This work reflected the findings of others who had also demonstrated permanent polyneuronal synapse formation on individual muscle fibers following "hyper reinnervation" with increased numbers of motor axons [28]. Kuiken *et al.* again examined how so called hyper reinnervation influenced outcome in the rat medial gastrocnemius muscle [10]. Increasing numbers of motor axons were recruited from motor nerves to nearby muscles to increase the reinnervation ratio from 1:1 to 12:1 (2.6, 3 and 5.4 times the number of motor axons were also employed in the experiment). Using 5.4 and 12 times the normal number of motor axons to reinnervate the muscle improved outcomes in terms of muscle mass preservation and muscle force generation were demonstrated[2]. Unfortunately no direct nerve count values were given in the distal nerve segments for this study, nor were any values of proximal axonal cross sectional area. Unlike the work by Taxt, as the percentage of motor neurons used to reinnervate the muscle was increased fewer motor neurons were able to functionally reinnervate the muscle. This lead to the author to question the how and why this phenomenon occurred,

[2] With a reinnervation ratio of 12:1, 84% of the muscle mass was preserved compared to 65% when the gastrocnemius was reinnervated by its native nerve. Similarly 84% of the force of the contralateral control muscle was achieved using this technique, compared to 57% in the indigenous nerve. These findings were significant. Using a 5.4:1 ratio 72% muscle mass was preserved with 70% of the contralateral muscle force produced. This finding was only a trend however.

with the postulated answer being that some motor neurons were able to reinnervate the majority of the muscle, establishing normal sized and enlarged motor units. With few denervated fibers left only a few other motor neurons were able to establish small motor units. Those motor neurons able to reinnervate normal and enlarged motor units were thought to have had some sort of advantage, the most likely cause of this being the distal nerve tracks. Schwann cells within the distal nerve tracks are thought to produce trophic factors that guide regenerating axons towards and through them [29] [30] [31]. Motor neurons regenerating through those tracks might have been guided directly to residual endplates giving them an advantage in the reinnervation process.

The reason for the failed improvement in function in the muscle transfers subject to the increased axonal input in this experiment is probably multi factorial, drawing on several of the above ideas and findings. The rf group muscles recovered function to an adequate level, comparable with other published series (see discussion Chapter 3). This suggests that poor technique was unlikely to be the cause of the "poor" outcome in the vl group. As demonstrated by Cederna *et al.* and others even following repair of a divided motor nerve to include all the original axonal content there is still a residual population of denervated fibres within the muscle contributing to a force deficit [1] [11]. It is known that motor neurons can expand their motor unit size to accommodate for axonal loss following nerve repair, and therefore theoretically expansion of motor unit size should comfortably accommodate for neuronal loss[3]. This, as detailed previously, is seldom the case however. Peripheral motor nerves contain both α (large – to the extrafusal muscle fibres) and γ (small – to the intrafusal muscle fibres) efferent motor neurons. They also contain afferent myelinated sensory fibres (I, II, and III [32]) [33]. It is known that denervated muscle fibres can be preserved or reinnervated (sensory neurotization) by sensory fibres, and it is likely that this is one of the factors influencing the residual population of non functional muscle fibres seen post nerve repair [34] [35].

In the 2 groups examined in this study, it is probable that motor axons crossed the neurorrhaphy and followed distal nerve tracks allowing reinnervation of the muscle with

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[3] This expansion of motor unit size is elegantly demonstrated by van der Meulen *et al.* where 14% of the normal amount of axons are able to recover 55% of the maximal tetanic force of the rat EDL muscle.

formation of normal or enlarged motor units. In both cases it is possible that the complete recruitment of muscle fibres with in the muscle grafts was unachievable by the motor neurons due to inappropriate sensory neurotization of some of the muscle fibers by afferent nerve fibres. Although increases in the reinnervating loads of motor axons have demonstrated improved outcomes, this has been in the face of extremes of hyper reinnervation (12 times the normal motor axon input). It is possible that such high numbers of motor axons decrease the likelihood of a sensory afferent fiber entering a distal nerve track (due to extreme competition from the motor axons) and that at these levels, it is possible to produce the significant benefits in terms of muscle function, though at lower levels of competition the effect is insufficiently magnified to produce improvements in force generation (witnessed by the relatively poor out comes seen in the groups subject to lower reinnervation ratios in the same study). Failure to establish a target organ results in eventual die back or pruning of axons, and for the reasons given above this may be the factor responsible for the similar axonal counts distally seen in both the rf and vl groups.

Under circumstances encountered in the peripheral nervous system, whilst it is highly likely that improved outcomes can be achieved following motor nerve repair, modest levels of hyper reinnervation would seem to fail to produce improvements over the levels of recovery seen with the indigenous (or a size matched) nerve. The postulated reasoning for this is outlined above. Whilst pragmatically it is unlikely that clinicians would be able to improve reinnervation ratios to the 12:1 level, the idea of motor axonal overload is still attractive. If the problem faced is indeed sensory afferent competition (it is unlikely that the size of the motor nerve branch to muscle itself is a limiting factor as Mackinnon *et al.* demonstrated that a motor nerve trunk can carry approximately 2.5 times its normal number of nerve fibres if required to do so) perhaps an increase in innervation ratio coupled with measures designed to improve reorganization and direction of nerve growth are the answer [21]. The use of a gap between the nerve endings (such a 5mm length of silicone tubing) has been shown to improve directional growth of motor axons – neurotropism [36] [37]. Despite the obstacles faced in producing improved results, as other authors and proponents of functional muscle transfer comment, the use of increased numbers of motor axons to reinnervate peripheral functional muscle transfers should, where possible be encouraged so long as it has no deleterious effect [38] [10].

4.5.2 Facial Reanimation Model

If the theory is correct that axonal size and number influences the number of muscle fibres reinnervated by a cranial nerve, then this theory may be used to explain the spectrum of results seen with facial reanimation surgery. As detailed previously in Chapter 1 section 1.5, sequential increases in the number of reinnervating motor axons may explain how too few (axons) reinnervating the muscle results in little or no movement, whilst the "correct" number results in muscle graft force production equivalent to that of the native musculature it is aiming to reproduce – the optimal result (spontaneous and symmetrical motion). Further increases in reinnervating axonal numbers would produce further increases in transferred muscle force beyond that of the musculature it is aiming to replace, and this would lead to the over activity or tightening described, especially when the muscle is deprived of proprioceptive innervation and therefore deprived of muscle tone regulation [39] [40] [41] [42] [43] [44].

The model employed in the current study allowed comparison of the effects of a large and small axonal load on the function of a standard sized muscle transfer. The ventral ramus of the buccal branch of the facial nerve offered approximately 300% of the total axonal cross sectional area of the marginal mandibular nerve (significantly different), and a difference in axonal number of approximately 5 times (again significant). At both time points the bb group demonstrated significant increases in total axonal area over the mm group in the segment of the motor nerve to the rectus femoris distal to the neurorrhaphy, demonstrating a 4 fold advantage at 6 months, and a 2.5 times advantage at 9 months. Axonal number was increased in the bb group by approximately 2 times at both time points, though this finding narrowly failed to achieve significance at the 6 month time point ($p = 0.053$). Whilst the bb group demonstrated little change in axonal number crossing the neurorrhaphy at either time point, the mm group demonstrated an increase of approximately 2 fold at each time point (this was significant and presumably represented increased terminal branching). Both groups demonstrated similar area fractions crossing the neurorrhaphy at each time point, with an increase in area fraction from approximately 64% to 84% between the 2 time points (presumably representing a mixture of increased numbers of axons crossing the neurorrhaphy coupled with axonal

maturation with increases in mean axonal size). With similar area fractions crossing the neurorrhaphy in both the mm and bb groups it would appear that the surgery and graft bed conditions did not offer an advantage to either group that might have influenced outcome.

The axonal profiles seen in the segment of the motor nerve to the rectus femoris distal to the neurorrhaphy in two groups are in agreement with the data from the physiological assessment of the muscles. At both time points the bb group developed increased force compared to that of the mm group, and that an increase in both axonal area and axonal number was noted in both groups between the 2 time points. The relationship between axonal number, total axonal cross section area and wcP_o force values are not however as easily discernable. In table 4.5.2.1 the aforementioned parameters are compared, with the incremental ratios between the groups for these parameters given. Although some concordance between increases in wcP_o and axonal number exist this trend is definitely not continued when axonal area is examined. This makes any comment on exact axon number and size matching for tailoring outcome impossible beyond that of stating that by increasing these parameters the overall physiological performance of a free muscle transfer is improved.

It is difficult to compare these results with data from other studies as little information exists on comparative studies of reinnervation ratios in the setting of the cranial nerves. Whilst it has been reported that functional reinnervation of the mimetic musculature of the rabbit has been achieved with only 12% of the original axonal number [45], the study in question did not examine physiological outcome of the reinnervated muscle, merely defining function as movement of quadratus labii superioris when the animal was tapped on the nose. In another study reinnervation of the scutuloauricularis muscle by its indigenous nerve (the eponymous branch of the VII nerve) or the buccal branch of the facial nerve produced no functional deficit 6 months post operatively. Both groups demonstrated no axonal loss across the neurorrhaphy, with no decrease in total axonal cross sectional area. There was no difference in the force produced by the muscle in either group [46]. This demonstrates a significant difference between the peripheral and central nervous system when reinnervating muscle, namely that minimal (or no) functional deficit is apparent in the muscle following repair of its motor nerve. This may well be explained by the fact that unlike the peripheral nervous system all of the large

myelinated fibers in the 7th cranial nerve are virtually exclusively motor, presumably because the proprioceptive afferent fibers of the facial musculature run in the trigeminal nerve, thus the potential for inappropriate sensory neurotization is dramatically reduced [47] [48]. The findings of a study by Frey *et al.* demonstrate similar results when portions of the rectus femoris muscle, or the pectoralis descendens muscle were transferred to the face and reinnervated using the motor nerve to scutuloauriculars, with all muscles recovering over 90% of their weight adjusted force production [4].

Table 4.5.2.1 : The ratio of observed increases in control and weight adjusted force, axonal cross sectional area (mm^2) and axonal number observed in the distal nerve segments of the motor branch to the rectus femoris muscle in the facial reanimation model groups. The relative ratio increases seen between consecutive groups (ordered sequentially in terms of physiological outcome) is given in the grey fields underneath each of the parameters detailed.

Group	cwP_o	Axonal Area	Axonal Number
6mm	20.78	0.06	1352
	1.13	2.3	1.1
9mm	23.56	0.14	1491
	1.19	1.78	1.43
6bb	28.27	0.25	2147
	1.4	1.44	1.41
9bb	39.74	0.36	3029

The limits of the ability of the facial nerve to reinnervate differing sized muscle transfers was examined by Giovanoli *et al.* [22]. Here a piece of rectus femoris double the size of the scutuloauricularis muscle was grafted to the scutuloauricularis bed and reinnervated using the nerve to the muscle. Almost double the force of the control scutuloauricularis muscle was produced by the rectus femoris, suggesting that this branch of the facial nerve possessed the ability to fully reinnervate double the number of muscle fibres that it was designed to. In the same experiment however, no further increases in force production were achievable by using a triple sized piece of rectus, suggesting that the capability of increased motor fibre reinnervation by this branch of the facial nerve was

limited to an expansion ratio of 2. This comparatively inferior ability of facial nerve motor axons to increase their reinnervating ratio in relation to that observed in peripheral motor neurons has been suggested previously [49]. This postulation may indeed be the case, since the axonal number and cross sectional area in the motor branch to the rectus femoris distal to the neurorrhaphy were not vastly different in the bb groups compared to the vl or rf groups, though the functional capacity of the muscle was around a third to a quarter of that seen in the peripheral models. Direct comparison of the 2 settings is difficult however as the location of the muscle in the face does not place it in a position of function (as opposed to the leg where it would still be actively participating in the process of locomotion), thus making these transfers more prone to disuse atrophy.

The high degree of correlation between the physiological outcomes observed and the axonal profiles of the motor branch to the rectus femoris distal to the neurorrhaphy in these groups serves to support the hypothesis put forward in this experiment. The nerve histomorphometry data implies that if the number of motor axons used to innervate a constant sized functional muscle transfer is increased the force generated by the transfer is also increased, and that for a given sized axonal input greater increases in force are observed between 6 and 9 months with larger axonal loads. These findings may go some way to explaining the clinical picture observed in the setting of facial reanimation.

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Chapter 5

Results

Intramuscular immunohistochemical studies using Protein Gene Product 9.5 and Neural Cell Adhesion Molecule to assess muscle reinnervation.

5.1 Introduction

Following division of the motor nerve to a muscle, deterioration of the distal axonal endoneural sheaths and phagocytosis of cellular debris occurs. Additionally the distal nerve undergoes Wallerian degeneration characterized by axonal loss and Schwan cell proliferation [1]. This results in the loss of neural tissue in the target organ (in this case muscle). The subsequent presence of any neural tissue within the muscle requires reinnervation from an external source.

The overall aim of this experiment was to assess how the outcome (defined as the tetanic contractural force generated by the muscle) of a functional muscle transfer was altered when differing sized nerves were used to reinnervate it. To this end a peripheral nerve reinnervation model was designed to mimic the situation seen in peripheral limb reconstruction, together with a cranial nerve model to simulate the conditions encountered in facial reanimation surgery. In each of these settings one group of muscle transfers was reinnervated using a smaller nerve, with a second reinnervated using a larger nerve. The exact experimental design is given in Chapter 2, section 2.1. The hypothesis of this work was that by using an increased axonal load to reinnervate a muscle, an improved functional outcome would be achieved.

The rational underlying the aforementioned theory is that force deficits noted post transfer are due to residual denervated muscle fibres and that an increased axonal load will reduce the size of this population.

Assessment of outcome in this study was defined as the force output of the muscle flap at 6 or 9 months post transfer, and that any differences noted between groups at these points, if the hypothesis were correct, could be attributable to the relative percentage population of denervated fibres within the muscle transfers. Using both muscle physiology and nerve histomorphometry measurements it is possible to demonstrate firstly muscle function (force output) and secondly the number and size of the axons distal to the site of nerve repair, thus providing indirect assessment of muscle innervation status. Neither of these parameters however directly demonstrates neural in growth into the muscle transfer, nor the proportion of residual denervated muscle fibres. In order to provide a means of directly assessing whether improved neural in growth into

muscle is achieved using larger nerves, and that this in turn results in a reduced number of denervated muscle fibres direct examination of the muscle tissue itself was undertaken using immunohistochemical markers.

5.1.1 Protein Gene Product 9.5

Protein Gene Product 9.5 (PGP 9.5) is a neuron specific cytoplasmic protein that can be used to identify neural tissue using immunohistochemical staining techniques [2]. The use of this marker has been employed previously as a measure of the degree of neural in growth and reinnervation of vascularised muscle transfers [3] [4]. The degree of tissue positivity for PGP 9.5 expression (ie: number nerve fibres present in the sample) has been shown to correlate with the preservation of muscle architecture and fibre size in muscle grafts [3]. Immunohistochemical staining for PGP 9.5 has also previously been used as a marker for the degree of reinnervation seen in functional muscle transfers [5].

The hypothesis of this thesis is that an increased axonal load in the distal segment of the motor nerve used to reinnervate a muscle transfer will improve functional outcome by reinnervation of an increased number of muscle fibres. If this were to be the case, intramuscular nerve regrowth patterns should therefore reflect the axonal load in the distal segment of the motor nerve. PGP 9.5 was used to demonstrate such patterns in the current study to allow comparison of levels of neural in growth into the muscle with axonal load in the distal segment of the motor nerve to the muscle.

5.1.2 Neural Cell Adhesion Molecule

Neural cell adhesion molecule (NCAM) is a cell surface glycoprotein that is abundant along the sarcolemma of embryologic, foetal and postnatal skeletal muscle. With innervation it becomes localized around the acetylcholine receptor at the neuromuscular junction and is specifically limited to this site in normal adult muscle [6]. It is therefore expressed in very low levels in skeletal muscle, though reappears within the muscle fibre cytoplasm if the muscle is subject to denervation, paralysis or muscle tenotomy [7] [8]. Following denervation, expression of NCAM is quickly up regulated, within as short a

period as 24 hours and is detectable for as long as 10 months following the insult [9] [6] [10].

The use of NCAM as a measure of denervation within a muscle that has undergone repair of its motor nerve has been described previously [11] [12] [9]. As with PGP 9.5, it is stained for using immunohistochemical methods. Its use within the current study is similar to that of PGP 9.5, as a direct marker of muscle innervation status, demonstrating the percentage of denervated muscle fibres within the muscle transfers.

5.2 Aims

To elucidate if differing sized, cranial reinnervating axonal loads used to reinnervate the rectus femoris muscle produce differing levels of neural tissue presence within muscle transfers at 6 and 9 months post operation.

To determine if increased levels of cranial reinnervating loads impacts on the percentage of residual denervated fibres within the muscle.

To examine whether increasing the axonal load reinnervating the rectus femoris muscle from a peripheral nerve results in an increased level of neural tissue presence within the muscle graft at 6 and 9months post transfer.

To identify if an increase in the axonal load of a reinnervating peripheral nerve is associated with a decrease in the level of denervated muscle fibres left within the transfer.

5.3 Methods

Forty eight male 3.0 – 3.5 kg New Zealand White rabbits were divided into 4 groups as detailed previously (Chapter 2, section 2.1). The rectus femoris muscle was used as a vascularised functional muscle transfer either orthotopically (coapted to its native nerve for its source of reinnervation) or heterotopically (coapted to the larger neighbouring

motor nerve to vastus lateralis) in the leg, to simulate the peripheral limb reconstruction scenario. The muscle was also transferred to the face where it was coapted to either the marginal mandibular branch or the larger ventral ramus of the buccal branch of the facial nerve to simulate the clinical facial reanimation setting. The transfers were then assessed physiologically and harvested for immunohistochemical assessment at either 6 or 9 months post procedure.

5.3.1 PGP 9.5

Following physiological assessment the transferred muscle flaps were harvested and cut across the transverse axis to give 6 equal sections. These were numbered according to their location with respect to the origin of the muscle (into the ilium)[1] (Figure 2.4.1). A standard section was taken from the mid point of the muscle (section number 4) and was processed as described in Chapter 2 section 2.4.2, and Appendix 5. Muscle sections examined were always taken from the proximal face of the tissue block. The whole section was examined by an independent observer (blinded as to the identity of the sections and experienced in the assessment of immunohistochemical staining) and the neural tissue present within the section assessed using a semi quantitative method. The contralateral rectus femoris muscle was assessed in the same manner and served as a control.

The degree of reinnervation of the section was scored using a modification of a previously published scoring system [4]: In this study reinnervation was scored +/- to +++ based on the presence of nerve fibres within the muscle observed. The definitions on the presence of PGP 9.5 stained nerve fibres used to score reinnervation by Kostacoglu *et al.* were "very few, few, moderate, many and abundant". In the present study this technique was modified so that nerve bundles were identified by staining with PGP 9.5 and graded according to their size. The scoring system is given in Table 5.4.1.1. The total score for each muscle was calculated and expressed as a percentage of that of the control muscle section to provide a "reinnervaton index" for the muscle

[1] In muscles transferred to the face, the origin of the muscle was easily identified as that nearest the vascular pedicle and anchored to the angle of the mandible.

(Figure 5.4.1.1). Comparison of reinnervation indices between groups was undertaken using unpaired t-test statistical analysis.

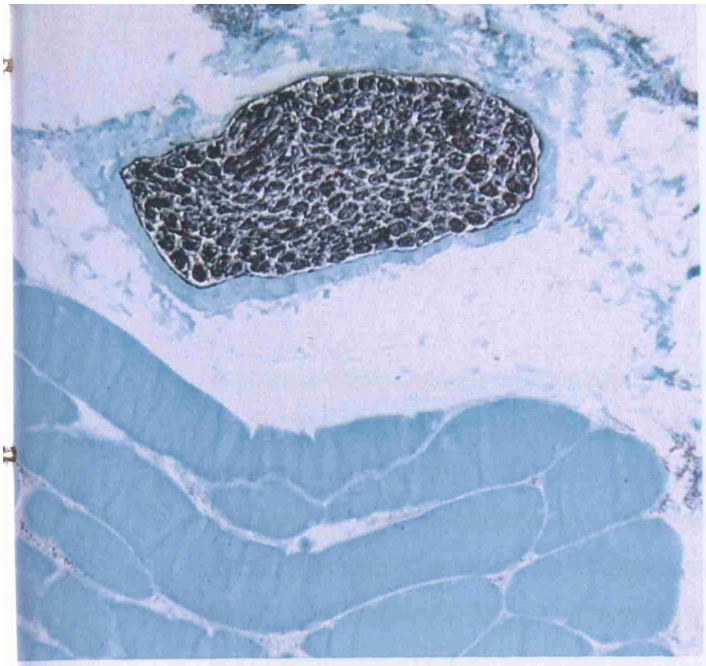
Table 5.3.1.1: Grading of PGP 9.5 stained nerve bundles within muscle sections.

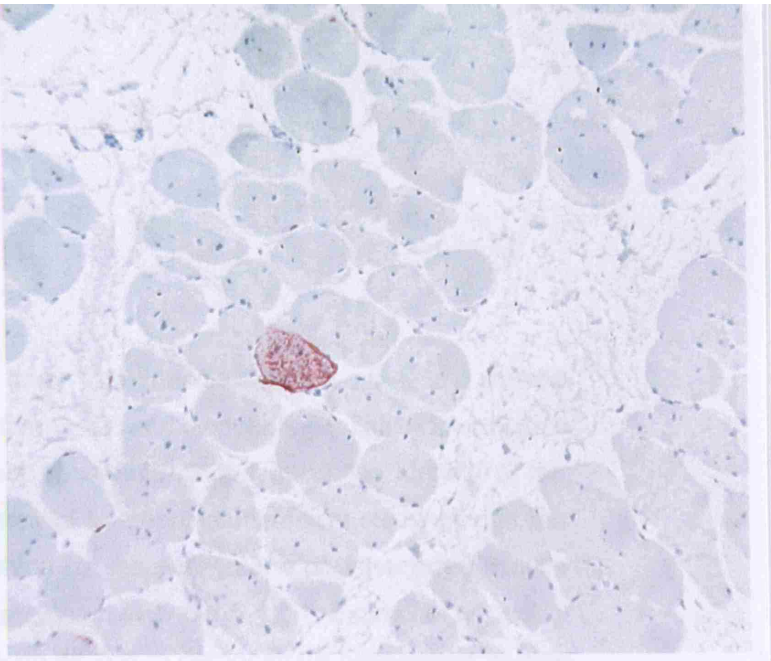
Nerve Bundle Diameter	Score
< 75 µm	+
75 – 150 µm	++
> 150 µm	+++

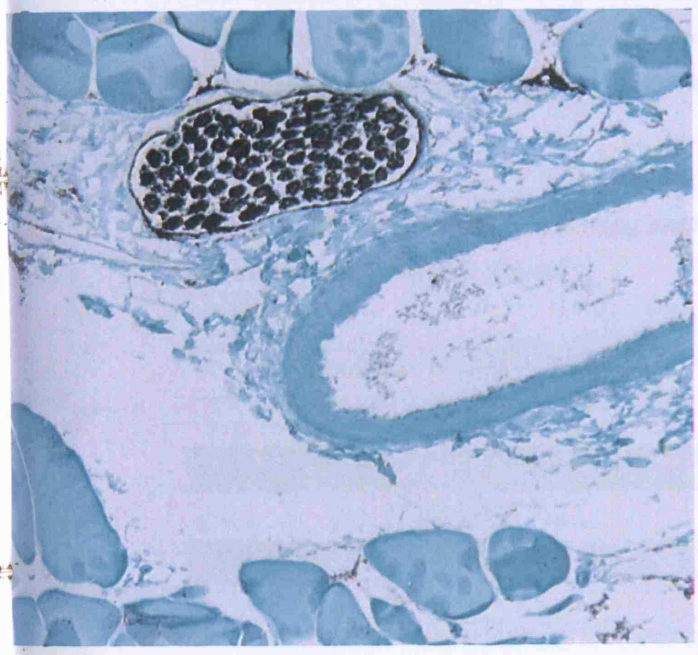
Figure 5.3.1.1: Reinnervation index calculation. Ao denotes the number of nerve bundles identified < 75 µm in diameter (+) in the muscle transfer, Bo those 75 - 150 µm (++) and Co those > 150 µm (+++). Ac, Bc and Cc denote the same values as above seen the control muscle.

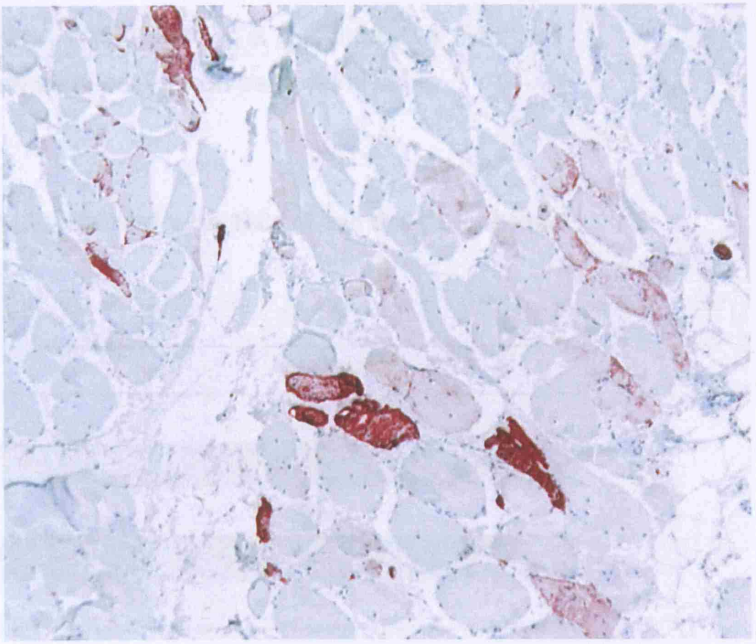
$$\text{Reinnervation index} = \left[\frac{(Ao \times 1) + (Bo \times 2) + (Co \times 3)}{(Ac \times 1) + (Bc \times 2) + (Cc \times 3)} \right] \times 100$$

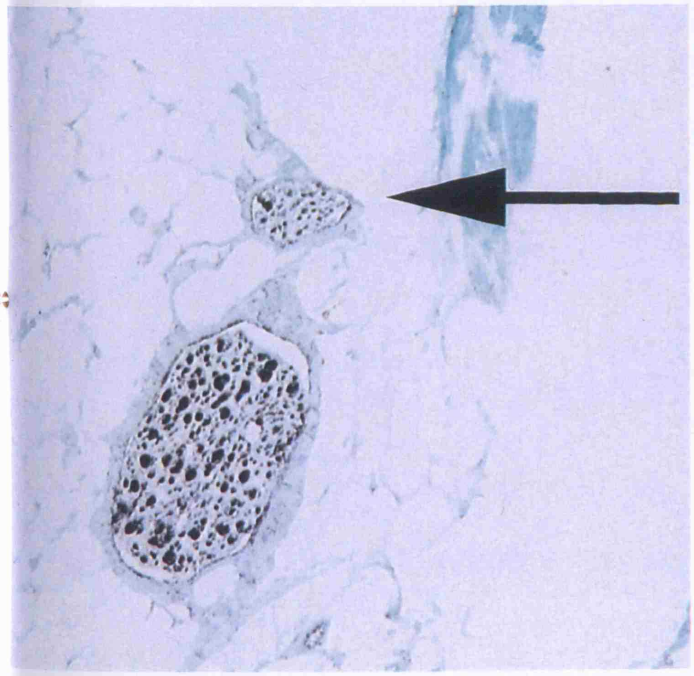
Figure 5.3.1.2: (overleaf) PGP 9.5 muscle specimen blocks. (Top, left) A muscle section demonstrating a large (+++) intramuscular nerve staining positive for PGP9.5 (blue/black). (Middle, left) A muscle section displaying a medium (++) intramuscular nerve staining positive for PGP 9.5. (Bottom, left) A muscle section staining containing small (+) (arrow) intramuscular nerve staining positive for PGP 9.5. Myocytes are stained green, all photomicrographs are at the same magnification. **NCAM muscle specimen blocks.** (Top, right) A control muscle block demonstrating an isolated NCAM +ve muscle fibre (red). Typically control specimens had less than 1/1000 NCAM +ve muscle fibres. (Middle, right) A muscle transfer 6 months post operatively. Modest amounts of NCAM +ve fibres are seen together with densely packed, reinnervated healthy myocytes (green). (Bottom, right) A muscle transfer 6 months post operatively revealing significant adipose replacement of myocytes (arrow), and moderate levels of NCAM +ve muscle fibres.

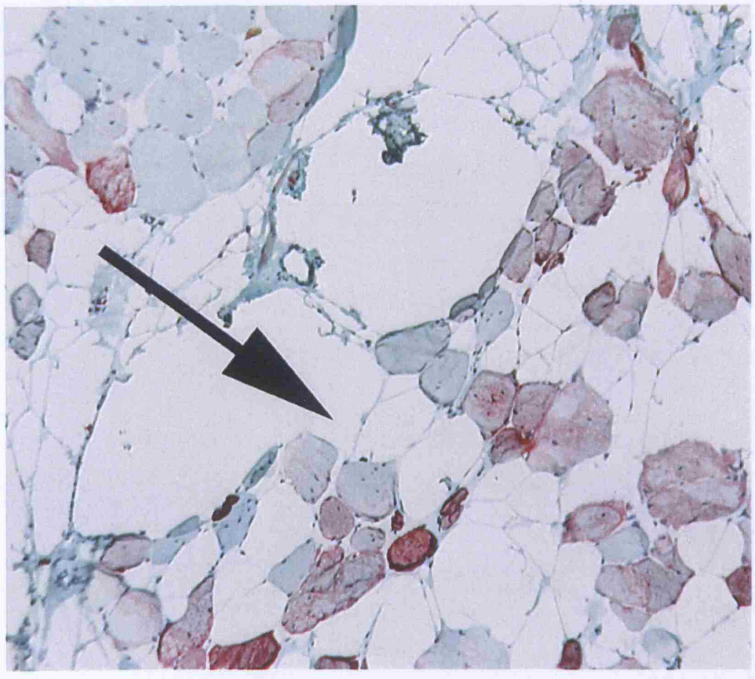












5.3.2 NCAM

A standardized muscle cross section was taken from muscle blocks 2 and 4, and stained for NCAM as described in Chapter 2 section 2.4.3. and Appendix 6. The whole muscle section was examined by an independent observer (blinded as to the identity of the section and experienced in the assessment of immunohistochemical staining) and the section scored in a semi quantitative fashion for the percentage of denervated muscle fibres. The details of the scoring system are given in Table 5.4.2.1. Examples of control and transferred muscle sections stained with NCAM to demonstrate denervated muscle fibres are shown in Figure 5.4.2. As the data was graded in a discontinuous fashion it was assessed using the Mann Whitney rank sum test for non parametric data.

Table 5.3.2.1 : Grading of muscle sections stained with NCAM for the percentage of denervated muscle fibres.

Percentage NCAM +ve fibres	Score
< 1%	1
< 10%	10
10 – 20%	20
20 – 40%	40
> 40%	50

5.4 Results

5.4.1 PGP 9.5

5.4.1.1 Facial Reanimation Model

The mean PGP 9.5 grading score for the 6 (month) marginal mandibular (mm) and 6 (month) buccal branch (bb) groups when expressed as a percentage of that of its contralateral control muscle (reinnervation index) was 63.2% (stdev 21.6, SEM 8.24) for the 6mm group and 111.6% (stdev 51.2, SEM 20.9) in the 6bb group. This finding was however insignificant, $p = 0.059$.

At the 9 month time point the mean reinnervation index value for the mm group was 50.6% (stdev 23.8, SEM 9.75). The mean value for the 9bb group was 99.5% (stdev 22.9, SEM 9.37), with the difference between the 2 groups being statistically significant, $p = 0.005$. There was no significant difference noted between time points within groups. The above data is illustrated in Figure 5.4.1.

5.4.1.2 Peripheral Reconstruction Model

The mean reinnervation index for the 6 month rectus femoris group (6rf) was 75.6% (stdev 45.6, SEM 18.6), compared to 87.2% (stdev 61.9, SEM 25.2) in the 6 month vastus lateralis group (6vl). There was no significant difference between these values $p = 0.71$.

Nine months post operatively the mean PGP 9.5 reinnervation index score for the rectus femoris group (9rf) was 124.0% (stdev 43.7, SEM 17.8). The mean value for the vastus lateralis group (9vl) was 143.0% (stdev 86.6, SEM 35.3), no significant difference being noted between the 2 groups $p = 0.81$ (Mann Whitney rank sum test used due to failure of normaility). Additionally there were no significant differences noted between time points within each group.

Figure 5.4.1: PGP 9.5 reinnervation indices for facial reanimation groupings. The groups are labeled on the x-axis, the reinnervation index on the y-axis. The mean value for each group is given within the column, the SEM value is detailed above the respective error bars. Significant values are denoted by an overhead line between the respective groups.

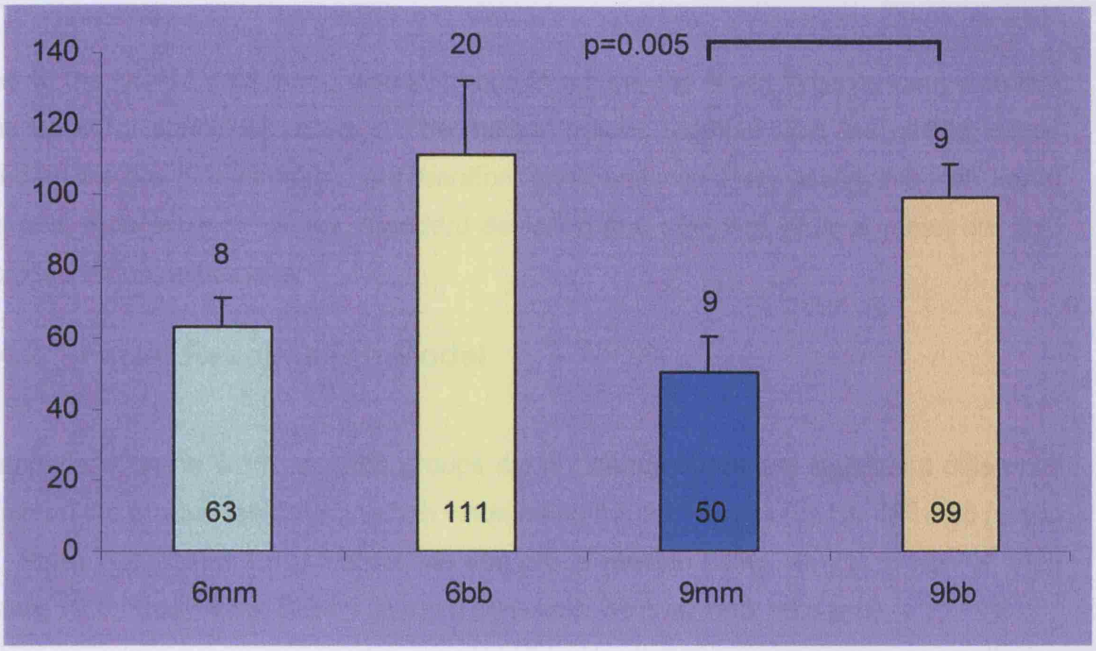
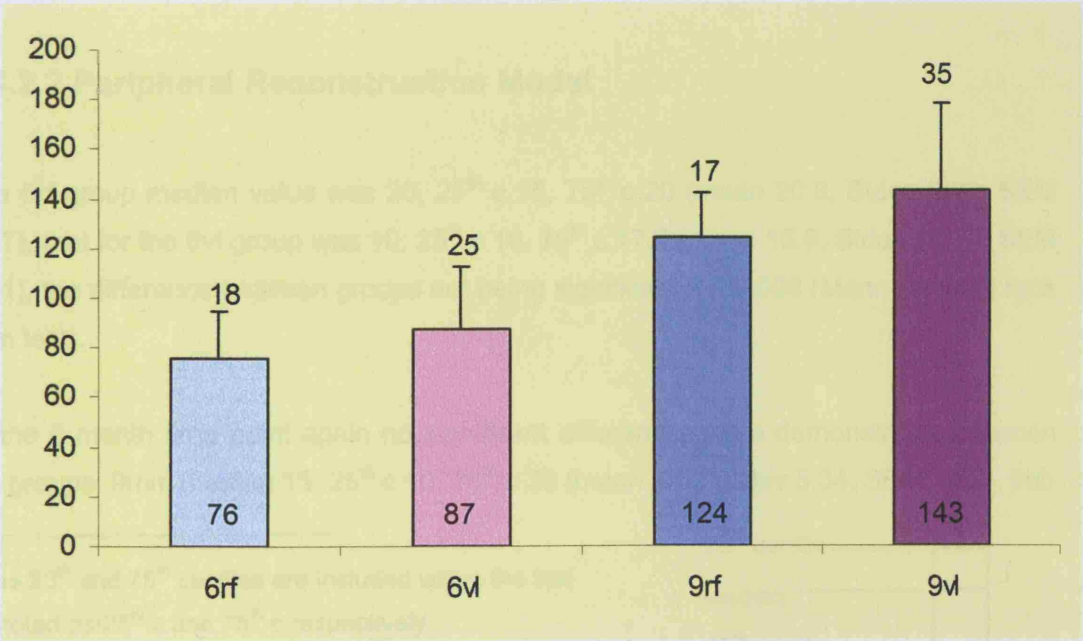


Figure 5.4.2: PGP 9.5 reinnervation indices for peripheral reconstruction groupings. The groups are labeled on the x-axis, the reinnervation index on the y-axis. The mean value for each group is given within the column, the SEM value is detailed above the respective error bars. Significant values are denoted by an overhead line between the respective groups.



5.4.2 NCAM

Due to the NCAM data being discontinuous in nature, the Mann Whitney rank sum test was used for statistical analysis. The median values together with the centile values used on the box plot diagrams[2] are therefore given as the primary descriptive statistics in the text, though mean values, standard deviation and standard error of mean are also included for completeness.

5.4.2.1 Facial Reanimation Model

Comparison of the 6mm and 6bb groups did not demonstrate any significant difference between the groups the 6mm median value being 15, 25th centile (c) 10, 75th c 20 (mean 15, Stdev 5.22, SEM 1.5,), that for the 6bb group median being 10, 25th c 10, 75th c 15 (mean 12.5, Stdev 4.53, SEM 1.3) p = 0.30 (Mann Whitney rank sum test).

The 9mm group demonstrated a median value of 20, 25th c 10, 75th c 40 (mean 25.8, Stdev 15.05, SEM 4.34), the 9bb group median being 10, 25th c 10, 75th c 10 (mean 11.6, Stdev 3.89, SEM 1.12). The difference between these groups was statistically significant p = 0.019 (Mann Whitney rank sum test). The above data is illustrated in figure 5.4.3.

5.4.2.2 Peripheral Reconstruction Model

The 6rf group median value was 20, 25th c 15, 75th c 20 (mean 20.8, Stdev 9.96, SEM 2.87), that for the 6vl group was 10, 25th c 10, 75th c 17.5 (mean 15.9, Stdev 13.30, SEM 4.61), the difference between groups not being significant p = 0.096 (Mann Whitney rank sum test).

At the 9 month time point again no significant differences were demonstrable between the groups: 9mm median 15, 25th c 10, 75th c 20 (mean 15.0, stdev 5.34, SEM 1.89), 9bb

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[2] The 25th and 75th centiles are included within the text denoted as 25th c and 75th c respectively.

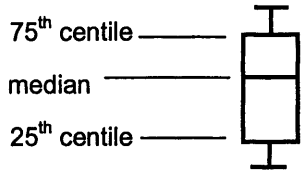


Figure 5.4.3: NCAM +ve muscle fibre percentages in facial reanimation groupings. The groups are labelled on the x-axis, the percentage of NCAM +ve fibres on the y-axis. The boxes represent the 25th and 75th centiles, with the median value shown as a line within the box. The 10th and 90th centiles are marked out by the whiskers. Outliers are marked by an asterisk. Significant values are denoted by an overhead line between the respective groups.

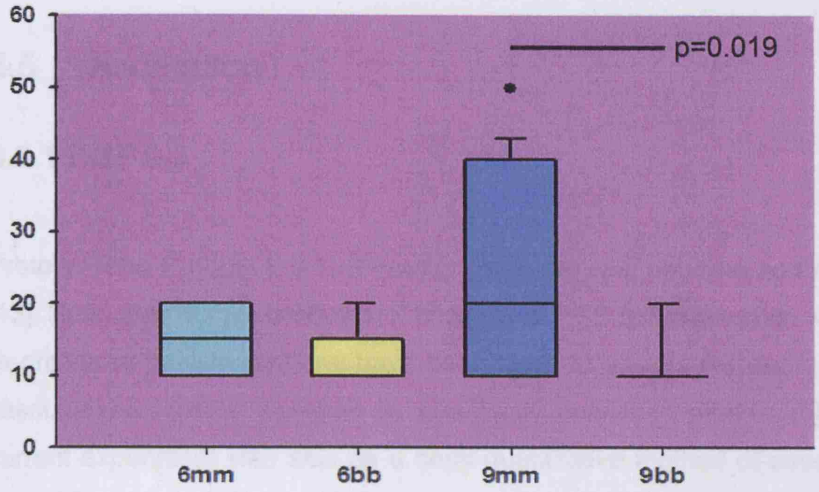
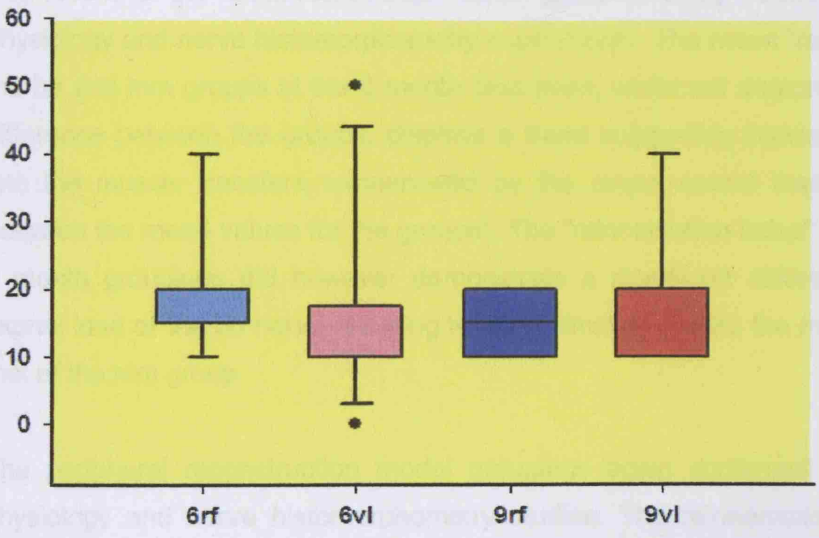


Figure 5.4.4: NCAM +ve muscle fibre percentages in peripheral reconstruction groupings. The groups are labelled on the x-axis, the percentage of NCAM +ve fibres on the y-axis. The boxes represent the 25th and 75th centiles, with the median value shown as a line within the box. The 10th and 90th centiles are marked out by the whiskers. Outliers are marked by an asterisk. Significant values are denoted by an overhead line between the respective groups.



median 20, 25th c 10, 75th c 20 (mean 20.0, stdev 10.44, SEM 3.01) p = 0.37 (Mann Whitney rank sum test). The above data is illustrated in Figure 5.5.4.

5.5 Discussion

5.5.1 PGP 9.5

Protein Gene Product 9.5 is a marker for vertebrate neurons and neuroendocrine cells [13]. Semi quantitative methods of analysis of PGP 9.5 expression within areas of neural re-growth in muscle sections have been used to assess the degree of reinnervation in vascularised muscle transfers in previously published studies [3] [4]. Its use in the current experiment was also as a semi quantitative method of assessing the degree of neural in growth following division and repair of the motor nerve to a muscle. It was intended to provide a direct assessment of neural in growth in to the muscle and to act as a corroboratory test for the physiological and nerve histomorphometry results detailed in Chapters 3 and 4. In an effort to optimize analysis of the muscle sections, an observer (blinded to the identification of the individual sections) experienced in immunohistochemical analysis was asked to score the sections.

The results of the facial reanimation model groups broadly confirm the findings of the physiology and nerve histomorphometry experiments. The mean "reinnervation index" of the bb and mm groups at the 6 month time point, whilst not demonstrating a significant difference between the groups, displays a trend suggesting improved neural in-growth into the muscle transfers reinnervated by the larger axonal load (a 75% difference between the mean values for the groups). The "reinnervation index" difference within the 9 month groupings did however demonstrate a significant difference with the larger axonal load of the bb nerve resulting in approximately double the mean "index" value of that of the mm group.

The peripheral reconstruction model groupings again confirmed the findings of the physiology and nerve histomorphometry studies. The reinnervation indices for these groups failed to demonstrate any significant difference between the mean values for either sized nerve at either time point. Unlike the 6 month facial reanimation muscles the

mean values within the peripheral reconstruction model muscle sections were closely grouped.

Drawing a broad correlation between the reinnervation indices of all the groups and the axonal content of the segments of the motor nerve to the rectus femoris muscle distal to the neurorrhaphy, it is possible to say that the axonal load in the distal segment of the nerve predicts axonal in growth into the muscle flap. As demonstrated in Chapter 4 the nerve histomorphometry findings reflect those of the physiology section of the study and therefore the PGP 9.5 analysis also concurs with the results of Chapter 3 (physiological assessment of the muscle transfers demonstrated no difference between the rf and vl groups at either time point). Comparison of the above results with the findings from previous studies is difficult because a detailed literature search reveals only a single previous experiment employing both nerve histomorphometry and PGP 9.5 measurements or PGP 9.5 and muscle physiology measurements [5]. The results however demonstrate good concordance between this and the aforementioned experiment in terms of correlation between PGP 9.5 analysis and muscle force measurements. In the other studies quoted in the text the use of PGP 9.5 was primarily to act as an indicator of nerve in-growth into the muscle transfer and correlate derived values to other morphological features such as degree of angiogenesis or muscle fibre size preservation [4] [12].

Overall, examination and comparison of physiology and nerve histomorphometry results shows good association with those of the PGP 9.5 analysis. This fact serves to endorse PGP 9.5 as a useful marker when examining for direct evidence of muscle reinnervation and weighs strongly in favour of its use in future studies.

5.5.2 NCAM

NCAM has been used as a marker for muscle denervation previously. In published studies NCAM muscle staining has been used in conjunction with physiological studies to determine the degree of muscle reinnervation seen following division and repair of the motor nerve to the muscle [12] [11]. Its increased expression has also been demonstrated following tenotomy of the rectus femoris muscle in the rabbit (without tendon repair) [9].

The NCAM immunohistochemical investigations conducted in the current work were undertaken to ascertain if it could be used to demonstrate the degree of residual denervation observed within a functional muscle transfer following restoration of neural input. In this way it could be used as an additional direct measure of whether functional outcome was related to the comparative populations of denervated muscle fibres.

As with the PGP 9.5 results, these findings broadly echo those of the physiology and nerve histomorphometry sections of the study (significant increases in force production and axonal content of the distal nerve segment in the bb group compared to the mm group, with no demonstrable differences between rf and vl groups). Specific findings within the groups studied show results similar to those found in the PGP 9.5 investigations; a significant difference demonstrable between the 9 month mm and bb groups, with fewer denervated fibres present in the bb group. The peripheral limb reconstruction groups did not show any differences amongst the groups. Direct interpretation of the NCAM score in terms of functional performance of the muscle transfers is difficult as the data is non parametric and grouped, unlike the physiology data. Furthermore whilst NCAM expression has be shown to correlate with muscle denervation (and potentially with disruption of the muscle excitation - coupling system responsible for myocytes contraction), direct correlation between expression and decrease in force production has not been previously established [9] [12].

In this current study the only significantly differing values, in the 9 month facial reanimation groupings, suggest that whilst the NCAM findings fit in with the general picture determined by the physiology, nerve histomorphometry and PGP 9.5 data, but as with previous reports, direct correlation between NCAM muscle status and muscle force

production is not possible. A 10% difference in denervated muscle fibre population between the mm and bb groups is unlikely to be responsible for the near 100% increase in control weight adjusted force output observed[3]. This finding agrees with those noted by other authors and underlines the need for caution when attempting to directly translate NCAM values in muscle force production values. The reasons for this are as yet unclear and as such suggest that it would be prudent to limit the role of NCAM to a secondary, corroboratory marker when aiming to directly assess muscle innervation status and attempting to relate it to functional outcome.

[3] One possibility is that the denervated muscle fibres present may represent a population of refractory fibres that whilst having failed to become reinnervated have also resisted atrophy and fatty degeneration (the majority of fibres already having being replaced by adipose). If this were the case the current method of assessment, which only examines intact myocytes and does not take into account adipose tissue within the epimyceum, would fail to demonstrate differences that may exist.

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Chapter 6

Results

Muscle Histomorphometry

6.1 Introduction

Muscle is composed of individual myocytes which belong to discrete motor units, each being innervated by a single motor neuron (under normal physiological circumstances). Within a motor unit all muscle fibres belong to single fibre type, the idea behind fibre types being that all fibres belonging to a particular type have similar functional properties [1]. Muscle fibre type specificity is conferred by the innervating motor neurone [2].

Normal muscle displays a mosaic appearance when the muscle fibres are stained according to their fibre types, due to the spatial distribution of the fibres of a single motor unit throughout a region of the tissue (ie; the fibres are not contiguous) [3]. With division of the neural supply to a muscle the ability to distinguish fibre type is lost [4]. Subsequent reinnervation of the muscle sees reestablishment of fibre type differentiation, though due to the nature of the reinnervation process when a nerve has been divided (as opposed to merely crushed), the individual muscle fibres of a motor unit are no longer distributed throughout a region of the muscle but tend to be clumped together, a phenomenon known as fibre type grouping [5].

As a result of reinnervation muscle fibres can undergo changes in not only fibre type specificity, but also in fibre size due to the atrophy/reinnervation process and as a direct consequence of a change in type specificity. It is known from previous studies that fibre type physiological properties differ (and as stated this is the basis for their group division), and that tetanic force output is greatest in type IIb fibres and least in type I fibres (type IIa residing in between the other 2 types). Similarly muscle fibre cross sectional area varies according to type with I < IIa <IIb [6]. It can be readily appreciated therefore that the functional capabilities of a muscle may be radically altered depending on the nerve used to reinnervate it.

A functional muscle transfer by its very nature undergoes reinnervation by a foreign neural source. By utilising a foreign nerve (as opposed to simply dividing and repairing the muscle's indigenous motor source) additional physiological imbalances are being placed on the muscle transfer which have a bearing on its final functional outcome. Alterations in muscle fibre type composition following reinnervation may well affect the performance of the muscle, and selection of the reinnervating nerve (where

circumstances allow) should aim to innervate the muscle transfer with motor neurons most suited to the task that the transfer is trying to achieve (eg; fast twitch motor neurons to recreate the flash mimectic function of zygomaticus major, rather than the use of the slow twitch fibres normally innervating buccinator) [7].

By analysing the effects of reinnervation using a foreign motor nerve on a standardised muscle transfer in a series of experimental settings, examining the effect on both fibre type composition and cross sectional area, the impact of muscle fibre make up and size on the physiological results can be determined.

6.2 Aims

Using the rectus femoris muscle as a functional muscle transfer the aims were:

To identify if muscle transfer in an orthotopic setting results in a change in muscle fibre type composition and mean fibre size.

To examine the effect of reinnervation with the foreign motor nerve to vastus lateralis (peripheral nerve), and to relate the findings of both of the aforementioned to the physiological outcomes seen.

To assess the effect of reinnervation using a large and a small branch of the facial nerve (cranial nerve) on muscle fibre type composition and size and determine the relationship (if any) to functional outcome.

To determine if significant differences are noted between all of the above and a control group of unoperated rectus femoris muscles.

6.3 Methods

Forty eight male 3.0 – 3.5 kg New Zealand White rabbits were divided into 4 groups as detailed previously (Chapter 2, section 2.1). The rectus femoris muscle was used as a vascularised functional muscle transfer placed orthotopically (coapted to its native nerve for its source of reinnervation) or heterotopically (coapted to the larger neighbouring motor nerve to vastus lateralis) in the leg, to simulate the peripheral limb reconstruction scenario. The muscle was also transferred to the face where it was coapted to either the marginal mandibular branch or the larger ventral ramus of the buccal branch of the facial nerve to simulate the clinical facial reanimation setting. The operated muscles were then assessed physiologically and harvested for immunohistochemical assessment at either 6 or 9 months post procedure as detailed previously in Chapter 2 sections 2.2 – 2.4

The muscles were harvested and cut across the transverse axis to give 6 equal sections. These were numbered according to their location with respect to the origin of the muscle (into the ilium)[1] (Figure 2.4.1). A standard section was taken from the mid-point of the muscle (section number 4) and was processed as described in Chapter 2 section 2.4.1, and Appendix 4. Muscle sections examined were always taken from the proximal face of the tissue block.

Four randomly chosen fields were captured, (x200 magnification) using a digital imaging system coupled to light microscope, by an operator blinded to the identity of the sections and experienced in imaged capture techniques. An average of 275 muscle fibres were counted, typed and sized for each muscle, this being taken as representative of the muscle as a whole. The percentage distribution of fibre types was calculated for each muscle, as was the relative muscle fibre cross sectional are for each fibre type. A panel of 6 randomly chosen unoperated rectus femoris muscles from the contralateral leg served as controls.

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[1] In muscles transferred to the face, the origin of the muscle was easily identified as that nearest the vascular pedicle and anchored to the angle of the mandible.

6.4 Results

A standardised muscle section was examined from each muscle transfer, in addition to which 6 randomly chosen control muscles were examined in the same way to provide control values. Each section was investigated as described in the methodology (sections 2.4 and 6.3). Mean fibre type percentages were calculated for each of the fibre types examined (types I / IIa / IIb) and mean fibre area determined. These measurements were then used for inter group comparison. The data was expressed in the form of error bar charts with 95% confidence intervals demonstrated about the mean. This is analogous to undertaking multiple t-tests between the groups (eg: 6mm vs 6bb, 9mm, 9b, control) as overlap between the confidence intervals indicates that the true value of the means may in fact be equal and therefore no significant difference between the groups examined exists. This form of data representation was chosen for ease of examination and interpretation, as overlap between confidence intervals of groups allows the observer to readily determine if there is any significant difference and it allows direct comparison of all groups at once. Figures 6.4.1 to 6.4.6 display the results seen in the peripheral reconstruction models and figures 6.4.7 to 6.4.12 those in the facial reanimation groupings. Table 6.4.1 details the mean value profiles for the peripheral reconstruction groups, table 6.4.2 those for the facial reanimation groups.

6.4.1 Peripheral Reconstruction Model

The mean type I fibre area for the 6 month rectus femoris group (6rf) was 1525 μm^2 with the bounds for 95% percent confidence intervals being zero (actual statistical value -726 μm^2) for the lower margin and 3778 μm^2 for the upper, with the range being 1680 μm^2, the standard deviation (stdev) and standard error of the mean (SEM) 906 μm^2 and 523 μm^2 respectively. The 6 month vastus lateralis group (6vl) displayed a mean fibre area of 1414 μm^2 with confidence interval boundaries of 161 μm^2 and 2667 μm^2 and a range of 1838 μm^2 (stdev 787 μm^2 and SEM 393 μm^2). The data for the 9 month rectus femoris group (9rf) was as follows: mean fibre area 698 μm^2, 95% confidence interval boundaries of zero (actual statistical value -1386 μm^2) and 2782 μm^2, range 328 μm^2, stdev 232 μm^2 and SEM 164 μm^2. The 9 month vastus lateralis group (9vl) had a mean type I fibre area of 907 μm^2 with upper and lower 95% confidence interval boundaries of

8.5 μm^2 and 1805 μm^2 respectively, range 1857 μm^2 (stdev 723 μm^2 and SEM 323 μm^2). Control mean fibre area was 1040 μm^2 with confidence interval boundaries of 694 μm^2 and 1386 μm^2, a range of 896 μm^2, stdev 329 μm^2 and SEM 134 μm^2.

The mean type IIa fibre area for the 6rf group was 507 μm^2 with the bounds for 95% percent confidence intervals being zero (actual statistical value -29.6 μm^2 for the lower margin and 1044 μm^2 for the upper, with the range being 404 μm^2, the standard deviation (stdev) and standard error of the mean (SEM) 216 μm^2 and 124 μm^2 respectively. The 6vl displayed a mean fibre area of 1110 μm^2 with confidence interval boundaries of 191 μm^2 and 2029 μm^2 and a range of 1238 μm^2 (stdev 577 μm^2 and SEM 288 μm^2). The data for the 9rf group was as follows: mean fibre area 725 μm^2, 95% confidence interval boundaries of zero (actual statistical value -3710 μm^2 and 5162 μm^2, range 698 μm^2, stdev 493 μm^2 and SEM 349 μm^2. The 9vl group had a mean type IIa fibre area of 952 μm^2 with upper and lower 95% confidence interval boundaries of 332 μm^2 and 1572 μm^2 respectively, range 1338 μm^2 (stdev 499 μm^2 and SEM 223 μm^2). Control mean fibre area was 1048 μm^2 with confidence interval boundaries of 749 μm^2 and 1347 μm^2, a range of 766 μm^2, stdev 285 μm^2 and SEM 116 μm^2.

The mean type IIb fibre area for the 6 month rectus femoris group (6rf) was 1271 μm^2 with the bounds for 95% percent confidence intervals being 951 μm^2 for the lower margin and 1592 μm^2 for the upper, with the range being 874 μm^2, the standard deviation (stdev) and standard error of the mean (SEM) 305 μm^2 and 124 μm^2 respectively. The 6 month vastus lateralis group (6vl) displayed a mean fibre area of 1456 μm^2 with confidence interval boundaries of 962 μm^2 and 1949 μm^2 and a range of 1101 μm^2 (stdev 470 μm^2 and SEM 192 μm^2). The data for the 9 month rectus femoris group (9rf) was as follows: mean fibre area 984 μm^2, 95% confidence interval boundaries of 448 μm^2 and 1519 μm^2, range 705 μm^2, stdev 336 μm^2 and SEM 168 μm^2. The 9 month vastus lateralis group (9vl) had a mean type IIb fibre area of 1298 μm^2 with upper and lower 95% confidence interval boundaries of 996 μm^2 and 1600 μm^2 respectively, range 719 μm^2 (stdev 27 μm^2 and SEM 117 μm^2). Control mean fibre area was 1839 μm^2 with confidence interval boundaries of 1334 μm^2 and 2343 μm^2, a range of 1358 μm^2, stdev 480 μm^2 and SEM 196 μm^2.

The proportion of type I muscle fibres seen in the 6rf group was 0.75%, the 95% confidence interval being 0.0% (actual statistical value -0.59%) to 2.1%, range 2.1%, stdev 1.2% and SEM 0.5%. These figures for the 6vl group were 0.81%, 0.0%(actual statistical value -0.08%), 1.7%, 1.7%, 0.8%, and 0.3% respectively. The mean type I fibre proportion in the 9rf group was 0.2%, with the lower boundary for the 95% confidence interval 0.0% (actual statistical value -0.2%), the upper 0.6% and range 0.6% (stdev 0.2%, SEM 1.2%). The mean proportion in the 9vl group was 2.2%, 0.4% and 4.0% the 95% confidence interval boundaries giving a range of 3.6% and stdev and SEM values of 1.7% and 0.7% respectively. The control type I fibre profile was mean 4.7% total fibre population, 2.8% to 6.7% the boundaries of the 95% confidence interval (range 3.9%), stdev 1.8% and SEM 0.7%.

The percentage of type IIa muscle fibres in the 6rf group was a mean of 0.8%, with a range of 1.8% between upper and lower 95% confidence interval boundaries of 0.0% (actual statistical value -0.2%) and 1.8% respectively, range 1.8%, (stdev 0.9%, SEM 0.3%). The 6vl group demonstrated a mean value of 3.6%, 95% confidence interval 0.4% - 6.7%, range 6.3%, sdtev 2.9%, SEM 1.2%. The data for the 9rf group demonstrated a mean of 0.8%, confidence interval boundaries of 0.0% (actual statistical value -0.6%) and 2.2%% (range 2.2%), stdev 0.9% and SEM 0.4%. The 9vl group had a mean type IIa fibre percentage of 1.4, with a range of 2.3% between the confidence interval boundaries of 0.2% and 2.5% (stdev 1.1%, SEM 0.4%). The control group showed a mean type IIa fibre proportion of 1.5%, 95% confidence interval boundaries of 0.5% and 2.5%, range 2.0%, stdev 0.9% and SEM 0.3%.

Type IIb muscle fibres represented a mean of 98.4% of the muscle fibre population in the 6rf group (95% confidence interval values 96.9% and 99.9%, range 3.0%) with stdev 1.4% and SEM 0.5%. The profile of the 6vl group was: mean 95.5%, 95% confidence intervals 91.9% and 99.1% (range 7.2%), stdev 3.4% and SEM 1.3%. The 9rf group had a mean type IIb fibre percentage of 98.9, the 95% confidence interval being 97.1% to 100% (actual statistical value 100.8%) (range 2.9%), stdev 1.1% and SEM 0.5%. The 9vl group percentages were similarly 96.3, 94.5 to 98.0 (range 3.5), 1.6 and 0.6. The values for the control group type IIb fibre proportions were: mean 93.6%, 95% confidence interval upper boundary 95.4%, lower boundary 91.8% (range 3.6%), stdev 1.6% and SEM 0.6%.

6.4.2 Facial Reanimation Model

The mean type I fibre area for the 6 month marginal mandibular group (6mm) was 591 μm^2 with the bounds for 95% percent confidence intervals being 476 μm^2 for the lower margin and 705 μm^2 for the upper, with the range being 300 μm^2, the standard deviation (stdev) and standard error of the mean (SEM) 109 μm^2 and 44.5 μm^2 respectively. The 6 month buccal branch group (6bb) displayed a mean fibre area of 663 μm^2 with confidence interval boundaries of 300 μm^2 and 1026 μm^2 and a range of 600 μm^2 (stdev 292 μm^2 and SEM 130 μm^2). The data for the 9 month marginal mandibular group (9mm) was as follows: mean fibre area 638 μm^2, 95% confidence interval boundaries of 421 μm^2 and 855 μm^2, range 600 μm^2, stdev 207 μm^2 and SEM 84.5 μm^2. The 9 month buccal branch group (9bb) had a mean type I fibre area of 916 μm^2 with upper and lower 95% confidence interval boundaries of 273 μm^2 and 1559 μm^2 respectively, range 1200 μm^2 (stdev 517 μm^2 and SEM 231 μm^2). Control mean fibre area was 1040 μm^2 with confidence interval boundaries of 517 μm^2 and 1386 μm^2, a range of 900 μm^2, stdev 329 μm^2 and SEM 134 μm^2.

The mean type IIa fibre area for the 6mm group was 787 μm^2 with the bounds for 95% percent confidence intervals being 517 μm^2 for the lower margin and 1056 μm^2 for the upper, with the range being 800 μm^2, the standard deviation (stdev) and standard error of the mean (SEM) 256 μm^2 and 104 μm^2 respectively. The 6bb group displayed a mean fibre area of 819 μm^2 with confidence interval boundaries of 405 μm^2 and 1233 μm^2 and a range of 1100 μm^2 (stdev 394 μm^2 and SEM 161 μm^2). The data for the 9mm group was as follows: mean fibre area 1179 μm^2, 95% confidence interval boundaries 103 μm^2 and 2256 μm^2, range 2800 μm^2, stdev 1025 μm^2 and SEM 418 μm^2. The 9bb group had a mean type IIa fibre area of 977 μm^2 with upper and lower 95% confidence interval boundaries of 480 μm^2 and 1474 μm^2 respectively, range 1000 μm^2 (stdev 400 μm^2 and SEM 179 μm^2). Control mean fibre area was 1048 μm^2 with confidence interval boundaries of 749 μm^2 and 1348 μm^2, a range of 800 μm^2, stdev 285 μm^2 and SEM 116 μm^2.

The mean type IIb fibre area for the 6 month marginal mandibular group (6mm) was 747 μm^2 with the bounds for 95% percent confidence intervals being 503 μm^2 for the lower margin and 990 μm^2 for the upper, with the range being 600 μm^2, the standard deviation

(stdev) and standard error of the mean (SEM) 232 μm^2 and 94.7 μm^2 respectively. The 6 month buccal branch group (6bb) displayed a mean fibre area of 995 μm^2 with confidence interval boundaries of 542 μm^2 and 1449 μm^2 and a range of 1200 μm^2 (stdev 432 μm^2 and SEM 176 μm^2). The data for the 9 month marginal mandibular group (9mm) was as follows: mean fibre area 912 μm^2, 95% confidence interval boundaries of 500 μm^2 and 1324 μm^2, range 800 μm^2, stdev 332 μm^2 and SEM 148 μm^2. The 9 month buccal branch group (9bb) had a mean type IIb fibre area of 1034 μm^2 with upper and lower 95% confidence interval boundaries of 523 μm^2 and 1545 μm^2 respectively, range 1000 μm^2 (stdev 411 μm^2 and SEM 184 μm^2). Control mean fibre area was 1839 μm^2 with confidence interval boundaries of 1334 μm^2 and 2344 μm^2, a range of 1400 μm^2, stdev 480 μm^2 and SEM 196 μm^2.

The proportion of type I muscle fibres seen in the 6mm group was 15.1%, the 95% confidence interval being 11.2% to 19.77%, range 8.57%, stdev 4.0% and SEM 1.65%. These figures for the 6bb group were 13.7%, 0.01%, 27.4%, 27.4%, 13.0%, and 5.3% respectively. The mean type I fibre proportion in the 9mm group was 11.3%, with the lower boundary for the 95% confidence interval 5.8%, the upper 16.8% and range 11.0% (stdev 5.2%, SEM 2.1%). The mean proportion in the 9bb group was 16.44%, 6.0% and 26.8% 95% confidence interval boundaries giving a range of 20.8% and stdev and SEM values of 8.3% and 3.7% respectively. The control type I fibre profile was mean 4.7% total fibre population, 2.8% to 6.7% the boundaries of the 95% confidence interval (range 3.9%), stdev 1.8% and SEM 0.7%.

The percentage of type IIa muscle fibres in the 6mm group was a mean of 6.6%, with a range of 7% between upper and lower 95% confidence interval boundaries of 3.1% and 10.1% respectively (stdev 3.3%, SEM 1.3%. The 6bb group demonstrated a mean value of 5.0%, 95% confidence interval 0% - 11.6% (lower boundary statistical value of -1.5%), range 11.6%, sdtev 6.2%, SEM 2.5%. The data for the 9mm group demonstrated a mean of 2.3%, confidence interval boundaries of 1.1% and 3.5% (range 2.4%), stdev 1.1% and SEM 0.4%. The 9bb group had a mean type IIa fibre percentage of 5.9, with a range of 8.5% between the confidence interval boundaries of 1.7% and 10.3% (stdev 3.2%, SEM 1.5%). The control group showed a mean type IIa fibre proportion of 1.5%, 95% confidence interval boundaries of 0.5% and 2.5%, range 2.0%, stdev 0.9% and SEM 0.3%.

Type IIb muscle fibres represented a mean of 77.8% of the muscle fibre population in the 6mm group (95% confidence interval values 71.6% and 84.0%, range 12.4%) with stdev 5.9% and SEM 2.4%. The profile of the 6bb group was: mean 81.2%, 95% confidence intervals 66.0% and 96.3% (range 30.3%), stdev 14.4% and SEM 5.8%. The 9mm group had a mean type IIb fibre percentage of 86.3, the 95% confidence interval being 80.2% to 92.4% (range 22.2%), stdev 5.8% and SEM 2.3%. The 9bb group percentages were similarly 77.5, 66.8 to 88.2 (range 21.4), 8.6 and 3.8. The values for the control group type IIb fibre proportions were: mean 93.6%, 95% confidence interval upper boundary 95.4%, lower boundary 91.8% (range 3.6%), stdev 1.6% and SEM 0.6%.

Table 6.4.1: Results peripheral reconstruction groups. The groups are denoted as rf (rectus femoris) or vl (vastus lateralis) prefixed by the time point at which they were harvested (ie; 6 or 9 months post operatively). Mean fibre type proportion is given for each experimental group, in percentages, for type I, II and IIb muscle fibres, together with a summary of descriptive statistics (stdev – standard deviation, SEM – standard error of mean). The minimum and maximum values noted for each fibre type within each group are also given. Type IIb mean muscle fibre area is also given (expressed in μm^2) for each experimental group.

group	mean	std. dev.	SEM	minimum	maximum
6rf type I	0.75 %	1.28	0.52	0.17	3.27
6rf type IIa	0.80 %	0.96	0.39	0	2.30
6rf type IIb	98.43 %	1.40	0.57	96.72	100
6rf IIb area	1271 μm^2	305	124	707	1581
6vl type I	0.81 %	0.86	0.35	0	2.22
6vl type IIa	3.62 %	2.97	1.21	0	6.87
6vl type IIb	95.56 %	3.41	1.39	91.85	100
6vl IIb area	1456 μm^2	470	192	1050	2151
9rf type I	0.20 %	0.25	0.12	0	0.53
9rf type IIa	0.80 %	0.92	0.46	0	1.61
9rf type IIb	98.9 %	1.16	0.58	97.84	100
9rf IIb area	984 μm^2	336	168	553	1259
9vl type I	2.25 %	1.72	0.70	0	5.03
9vl type IIa	1.40 %	1.13	0.46	0	3.03
9vl type IIb	96.33 %	1.66	0.68	94.92	98.93
9vl IIb area	1298 μm^2	287	117	911	1631
Control type I	4.79 %	1.85	0.75	2.68	7.65
Control type IIa	1.56 %	0.92	0.37	0.47	3.06
Control type IIb	93.64 %	1.68	0.68	90.90	95.09
Control IIb area	1839 μm^2	480	196	1388	2745

Table 6.4.2: Results facial reanimation groups. The groups are denoted as mm (marginal mandibular) or bb (buccal branch) prefixed by the time point at which they were harvested (ie; 6 or 9 months post operatively). Mean fibre type proportion is given for each experimental group, in percentages, for type I, II and IIb muscle fibres, together with a summary of descriptive statistics (stdev – standard deviation, SEM – standard error of mean). The minimum and maximum values noted for each fibre type within each group are also given. Type IIb mean muscle fibre area is also given (expressed in μm^2) for each experimental group.

group	mean	std. dev.	SEM	minimum	maximum
6mm type I	15.51 %	4.06	1.65	10.13	19.56
6mm type IIa	6.62 %	3.37	1.37	3.37	11.49
6mm type IIb	77.86 %	5.92	2.41	69.73	84.48
6mm IIb area	747 μm^2	232	94.7	511	1100
6bb type I	13.73 %	13.07	5.33	0	31.59
6bb type IIa	5.02 %	6.27	2.56	1.25	17.17
6bb type IIb	81.24 %	14.43	5.89	65.65	98.75
6bb IIb area	995 μm^2	432	176	463	1640
9mm type I	11.36 %	5.20	2.12	6.58	17.74
9mm type IIa	2.32 %	1.13	0.46	1.01	3.71
9mm type IIb	86.31 %	5.80	2.37	79.03	92.40
9mm IIb area	912 μm^2	332	148	639	1490
9bb type I	16.44 %	8.35	3.73	5.52	28.14
9bb type IIa	5.97 %	3.42	1.53	1.71	11.18
9bb type IIb	77.57 %	8.62	3.85	65.30	88.95
9bb IIb area	1034 μm^2	411	184	668	1700
Control type I	4.79 %	1.85	0.75	2.68	7.65
Control type IIa	1.56 %	0.92	0.37	0.47	3.06
Control type IIb	93.64 %	1.68	0.68	90.90	95.09
Control IIb area	1839 μm^2	480	196	1388	2745

Table 6.4.3: Muscle fibre cross sectional area means and confidence interval values. All values are in μm^2 with the mean values as indicated and upper and lower values the respective 95% confidence interval limits.

Type I	6rf	6vl	9rf	9vl	control
Upper	3778	2667	2782	1805	1386
Mean	1525	1414	698	907	1040
Lower	726	161	0	8.5	694

Type IIa	6rf	6vl	9rf	9vl	control
Upper	1044	2029	5162	1572	1347
Mean	507	1110	725	952	1048
Lower	0	191	0	332	749

Type IIb	6rf	6vl	9rf	9vl	control
Upper	1592	1949	1519	1600	2343
Mean	1217	1456	984	1298	1839
Lower	951	962	448	996	1334

Type I	6mm	6bb	9mm	9bb	control
Upper	705	1026	855	1559	1386
Mean	591	663	638	916	1040
Lower	476	300	421	273	695

Type IIa	6mm	6bb	9mm	9bb	control
Upper	1056	1233	2256	1474	1348
Mean	787	819	1179	977	1048
Lower	517	405	103	480	749

Type IIb	6mm	6bb	9mm	9bb	control
Upper	990	1449	1324	1545	2344
Mean	747	995	912	1034	1839
Lower	503	542	500	523	1334

Figure 6.4.1: Type I muscle fibre type distributions in the peripheral reconstruction model. The muscle composition in terms of fibre type is given as a percentage on the y-axis. The groupings are detailed on the x-axis. The mean fibre type percentage value is indicated by the marker (and given in figures next to it), the error bars are the 95% confidence intervals about the mean.

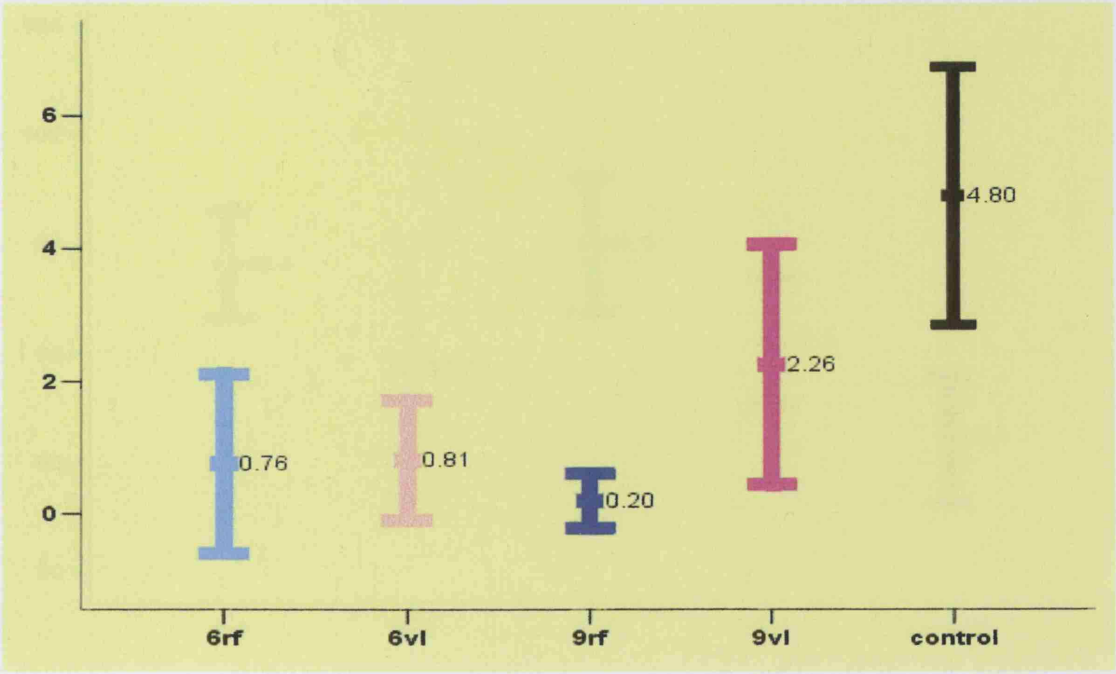


Figure 6.4.2: Type IIa muscle fibre type distributions in the peripheral reconstruction model. The muscle composition in terms of fibre type is given as a percentage on the y-axis. The groupings are detailed on the x-axis. The mean fibre type percentage value is indicated by the marker (and given in figures next to it), the error bars are the 95% confidence intervals about the mean.

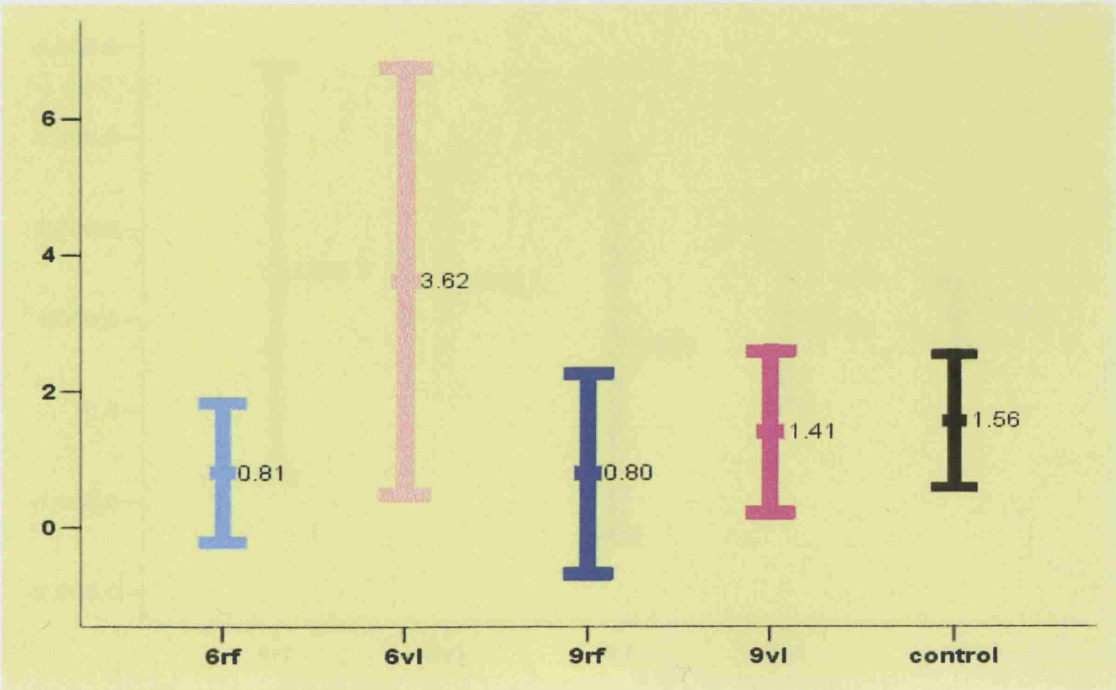


Figure 6.4.3: Type IIb muscle fibre type distributions in the peripheral reconstruction model. The muscle composition in terms of fibre type is given as a percentage on the y-axis. The groupings are detailed on the x-axis. The mean fibre type percentage value is indicated by the marker (and given in figures next to it), the error bars are the 95% confidence intervals about the mean.

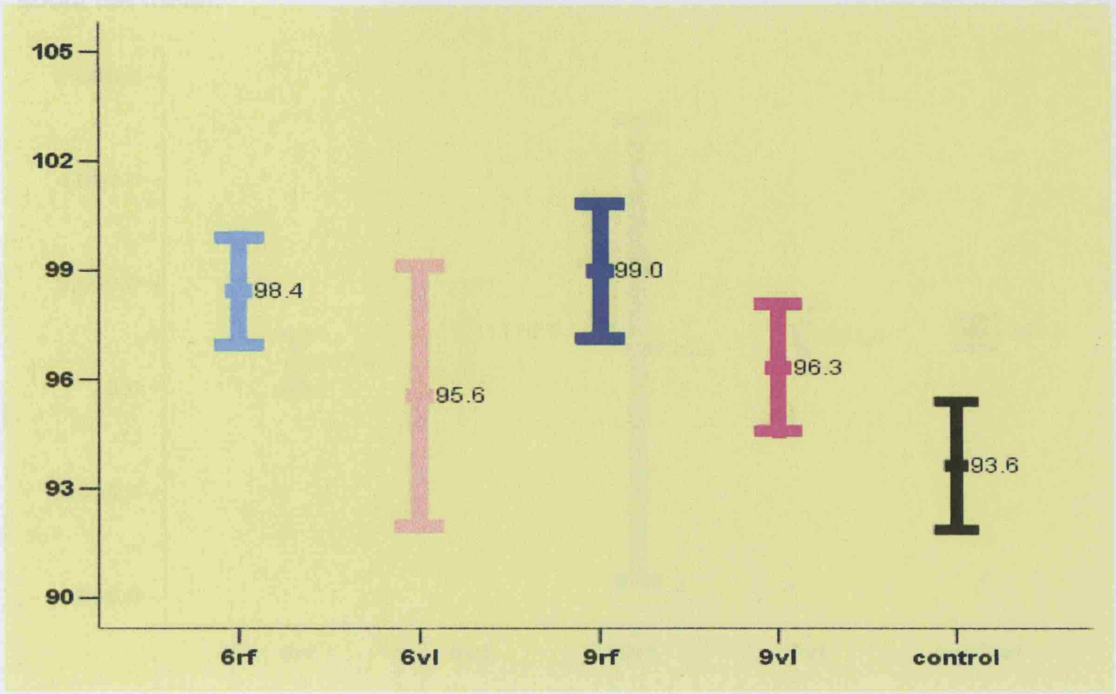


Figure 6.4.4: Type I muscle fibre type cross sectional area values in the peripheral reconstruction model. The fibre area is given on the y-axis in μm^2. The groupings are detailed on the x-axis. The mean fibre area value is indicated by the marker (and given in figures next to it), the error bars are the 95% confidence intervals about the mean.

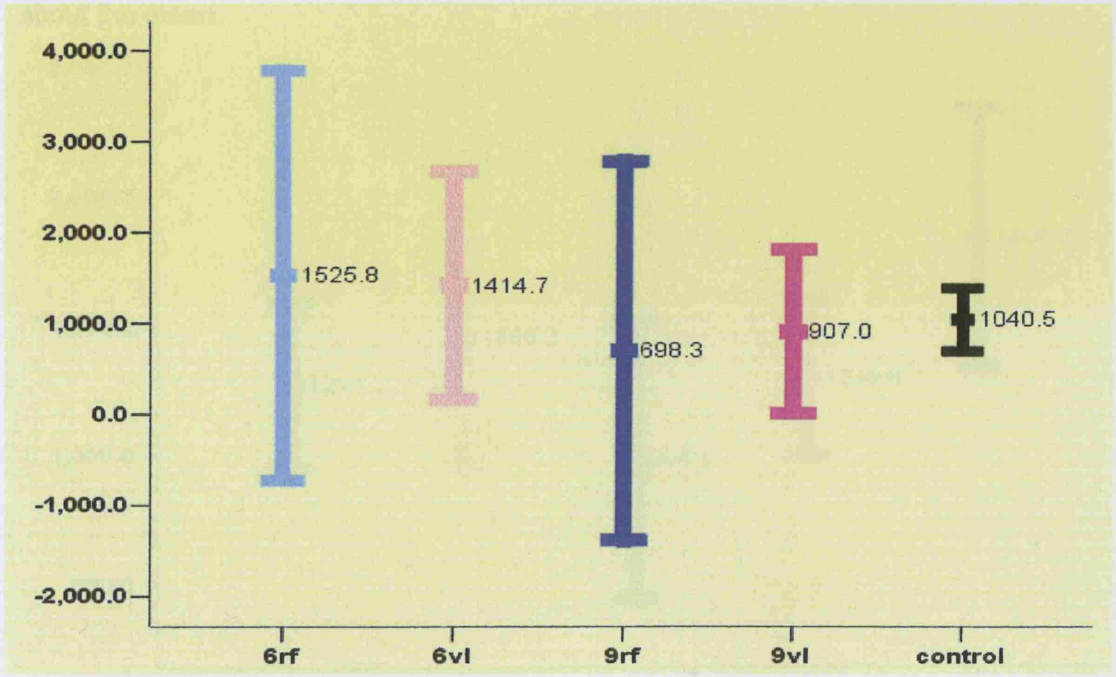


Figure 6.4.5: Type IIa muscle fibre type cross sectional area values in the peripheral reconstruction model. The fibre area is given on the y-axis in μm^2. The groupings are detailed on the x-axis. The mean fibre area value is indicated by the marker (and given in figures next to it), the error bars are the 95% confidence intervals about the mean.

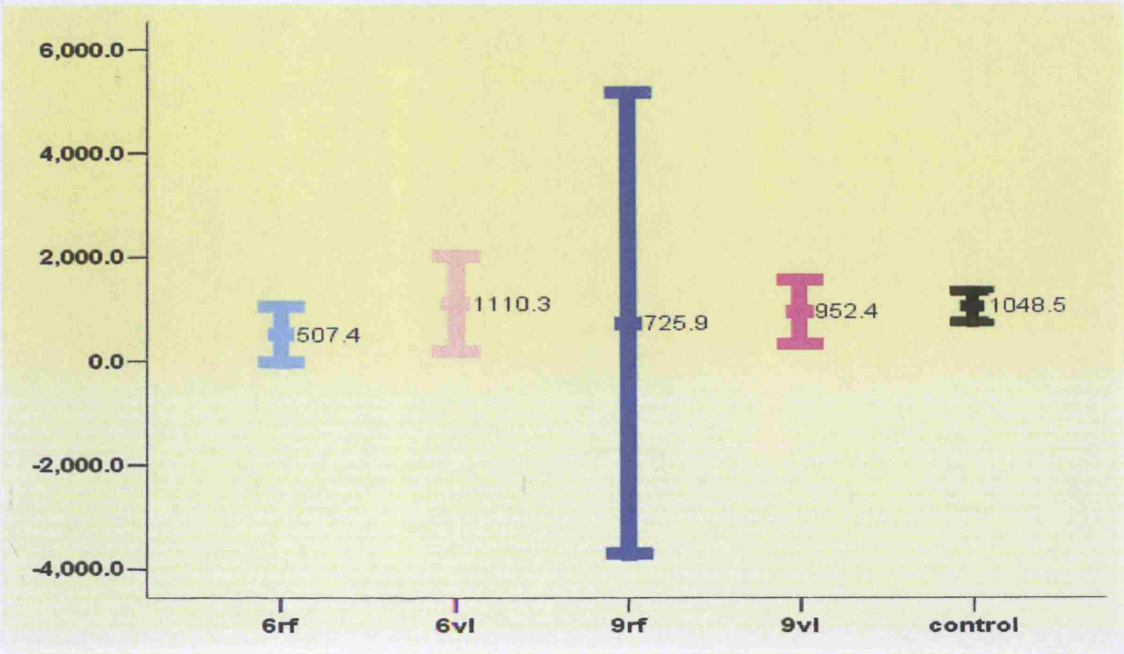


Figure 6.4.6: Type IIb muscle fibre type cross sectional area values in the peripheral reconstruction model. The fibre area is given on the y-axis in μm^2. The groupings are detailed on the x-axis. The mean fibre area value is indicated by the marker (and given in figures next to it), the error bars are the 95% confidence intervals about the mean.

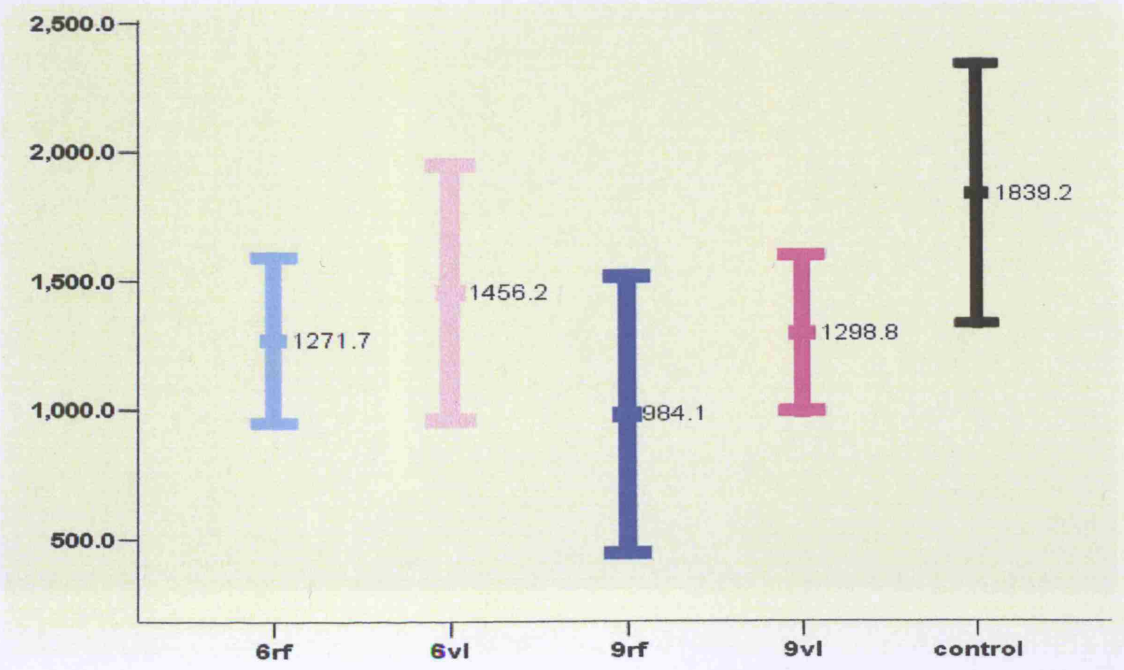


Figure 6.4.7: Type I muscle fibre type distributions in the facial reanimation model. The muscle composition in terms of fibre type is given as a percentage on the y-axis. The groupings are detailed on the x-axis. The mean fibre type percentage value is indicated by the marker (and given in figures next to it), the error bars are the 95% confidence intervals about the mean.

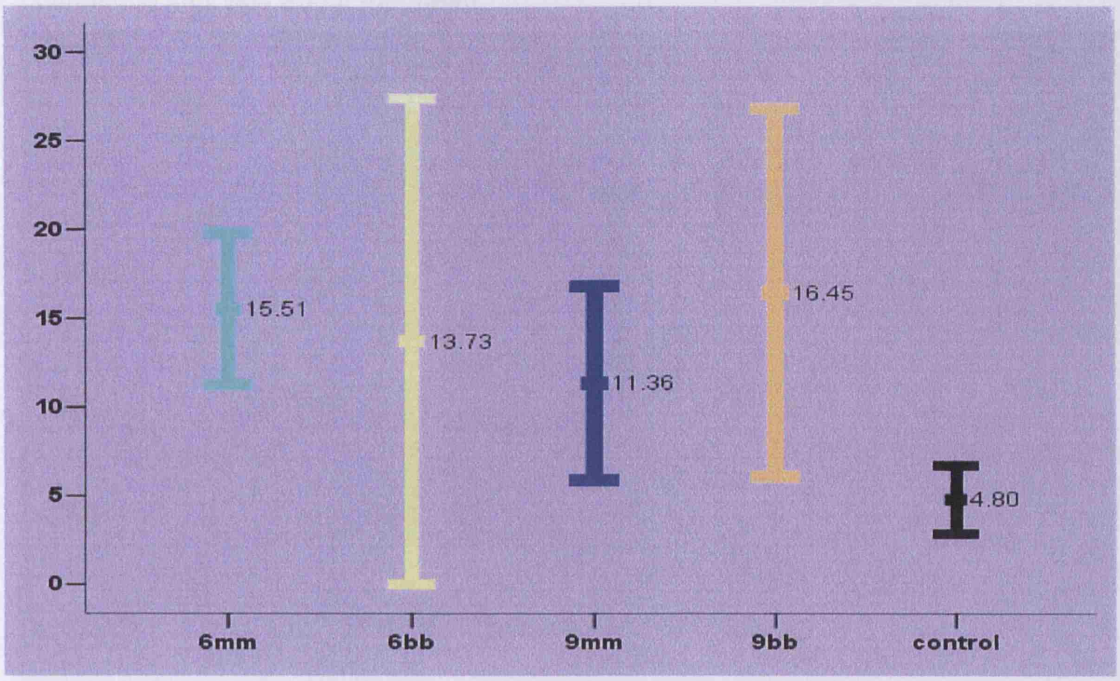


Figure 6.4.8: Type IIa muscle fibre type distributions in the facial reanimation model. The muscle composition in terms of fibre type is given as a percentage on the y-axis. The groupings are detailed on the x-axis. The mean fibre type percentage value is indicated by the marker (and given in figures next to it), the error bars are the 95% confidence intervals about the mean.

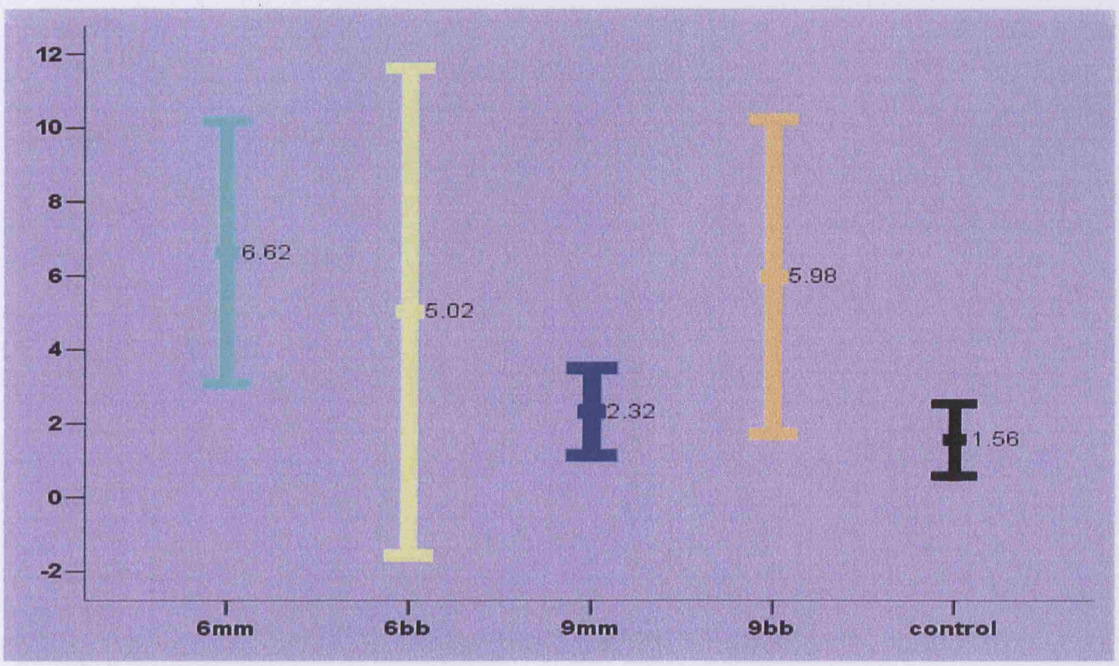


Figure 6.4.9: Type IIb muscle fibre type distributions in the facial reanimation model. The muscle composition in terms of fibre type is given as a percentage on the y-axis. The groupings are detailed on the x-axis. The mean fibre type percentage value is indicated by the marker (and given in figures next to it), the error bars are the 95% confidence intervals about the mean.

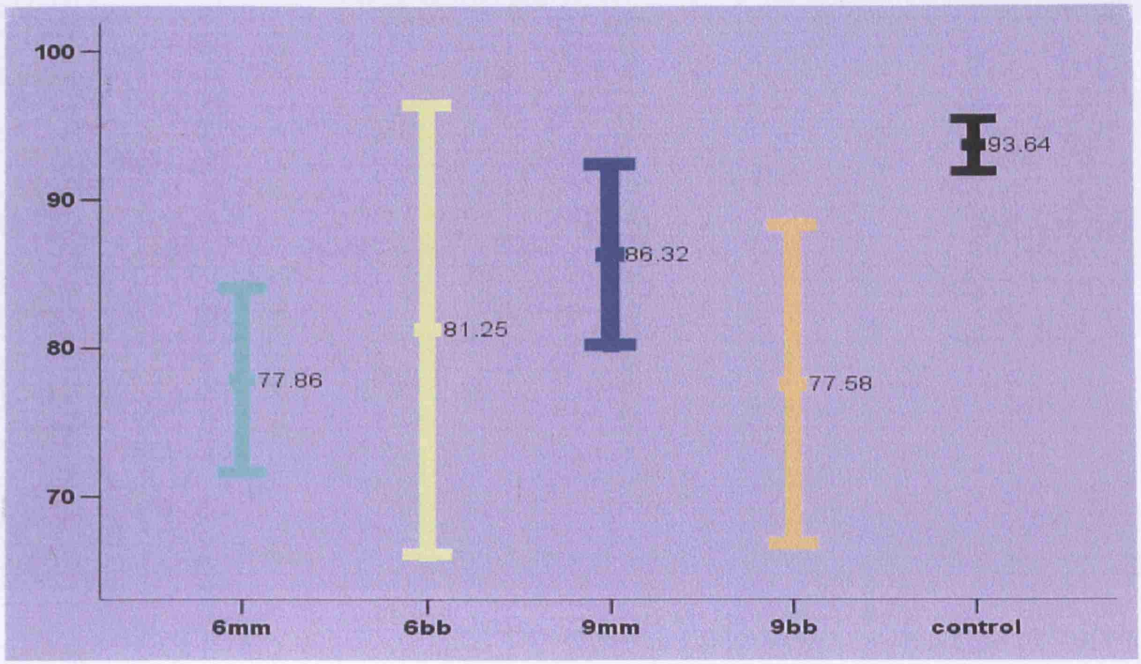


Figure 6.4.10: Type I muscle fibre type cross sectional area values in the facial reanimation model. The fibre area is given on the y-axis in μm^2. The groupings are detailed on the x-axis. The mean fibre area value is indicated by the marker (and given in figures next to it), the error bars are the 95% confidence intervals about the mean.

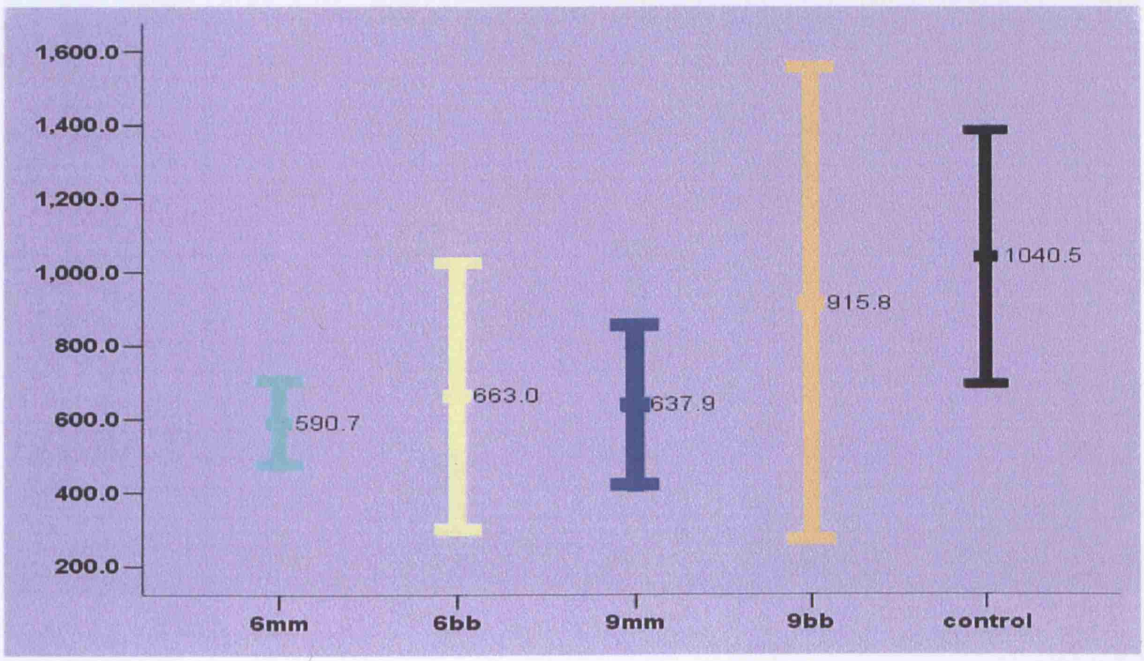


Figure 6.4.11: Type IIa muscle fibre type cross sectional area values in the facial reanimation model. The fibre area is given on the y-axis in μm^2. The groupings are detailed on the x-axis. The mean fibre area value is indicated by the marker (and given in figures next to it), the error bars are the 95% confidence intervals about the mean.

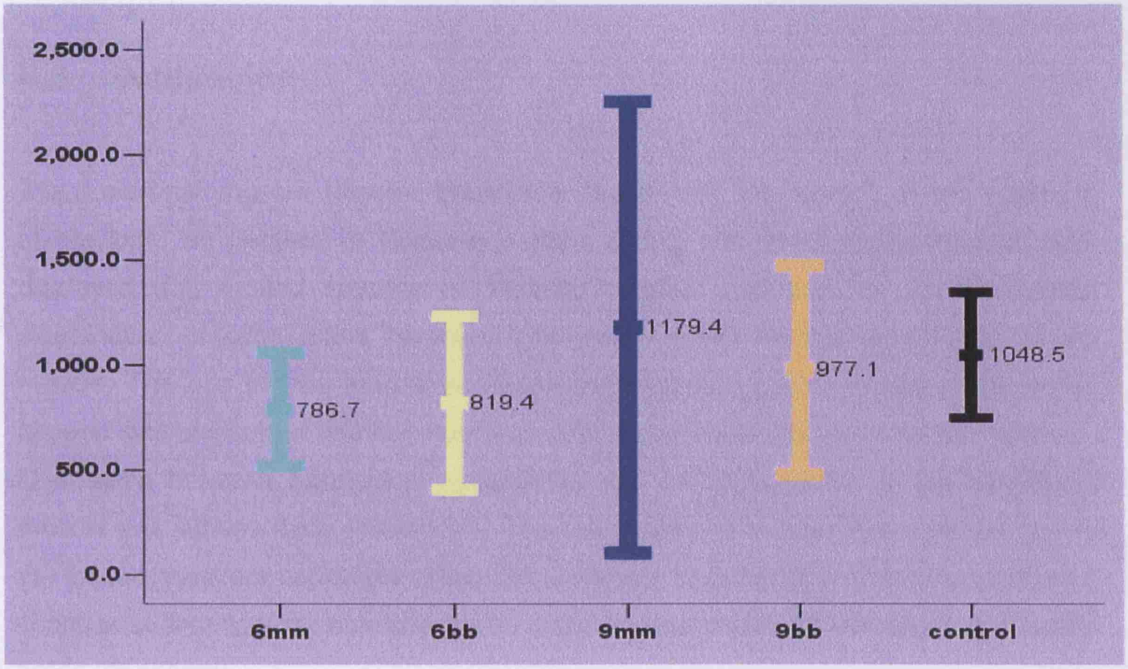
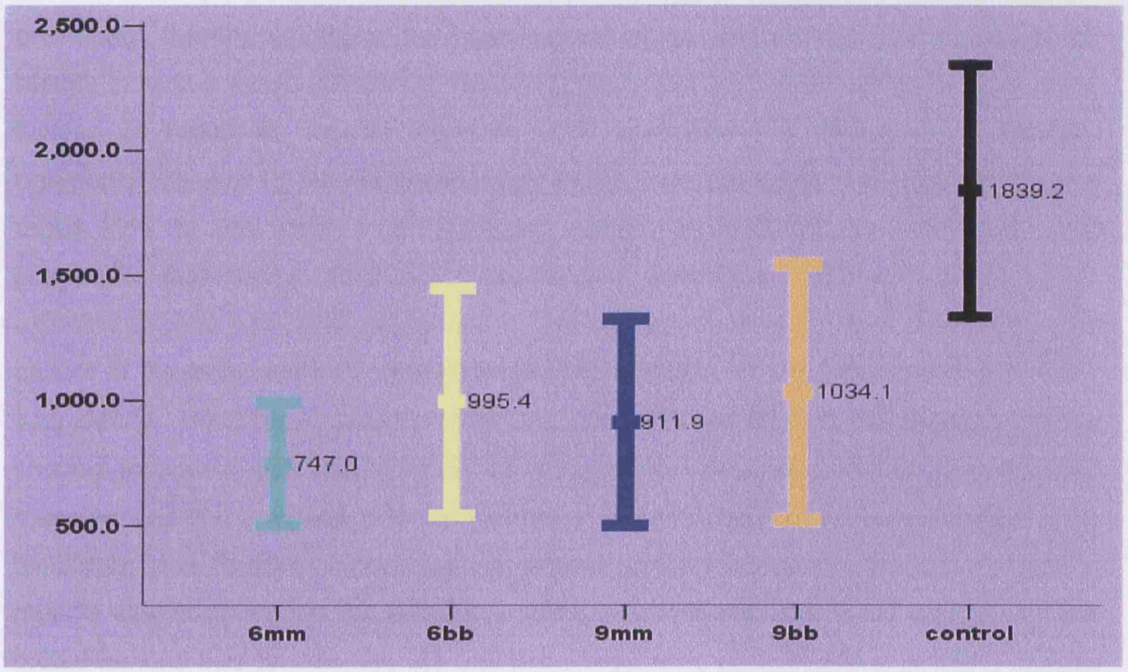


Figure 6.4.12: Type IIb muscle fibre type cross sectional area values in the facial reanimation model. The fibre area is given on the y-axis in μm^2. The groupings are detailed on the x-axis. The mean fibre area value is indicated by the marker (and given in figures next to it), the error bars are the 95% confidence intervals about the mean.



6.5 Discussion

The functional muscle transfer became a reality with the advent of the operating microscope, as detailed in Chapter 1. Prior to this non vascularised muscle grafts displayed only limited success in attaining function post transfer and histological examination of such grafts revealed breakdown of the internal architecture of the muscle. The use of free functional muscle transfers with microvascular anastomoses allowed preservation of internal muscle structure and an avoidance of central necrosis. Correlation between histological appearance and functional ability of the transferred muscle was subsequently established. The examination of muscle fibre size and type in the muscle transfers performed within this study was undertaken therefore to establish if changes in fibre type composition or size could be responsible for alterations in outcome noted (or absent).

6.5.1 Peripheral Reconstruction Model

The use of the rectus femoris muscle as a functional muscle transfer similar to the manner employed in this current experiment has been reported by several authors previously, thereby validating the experimental model and providing data with which results from this study can be compared [8] [9] [10] [11] [12] [13]. In his original study looking at functional muscle transfers Frey examined the effects of (essentially orthotopic) transfer of the rectus femoris muscle from the left to the right thigh in the rabbit [11]. In this work a standardised mid-muscle section was examined using enzymatic techniques (NADH-diaphorase and actomyosin ATPase reactions) to examine muscle fibre type composition. The authors commented that "the histological picture of the cross sections of the transplanted muscles reflected the regained function very clearly." However it was also noted that overall muscle fibres in the transplant group showed reduced mean values for area and perimeter when compared to normal rectus muscles, and that a reduction in the number of Type I muscle fibres was also seen [11]. Guelinckx and Faulkner under took a related experiment where the rectus femoris muscle was compared to the latissimus dorsi, with both muscles being grafted into the rectus femoris bed (in this way the authors compared a parallel and a pennate muscle post functional muscle transfer). Examining 150 muscle fibres in cross section within

each transferred muscle (taken to be representative) it was noted that fibre cross sectional area decreased for both the rectus and latissimus muscles (by 30% and 36% respectively, statistically significant). In addition to this significant changes were noted in fibre type composition in the latissimus muscle transfers when compared to those of the rectus (both control and transferred muscles) and the control latissimus muscles. The noted increase in Type I muscle fibres in the latissimus transfers (33% total fibre population compared to 17% in the latissimus muscles, 3% in the rectus muscles and 2% in the rectus transfers) was postulated by the authors to be a result of the structural and functional differences between the latissimus and rectus muscles resulting in an adaptive response by the latissimus muscle transfers to accommodate to the required muscle contraction velocity at the recipient site in the thigh compartment [10].

Examination of the impact of 1 stage or 2 stage reinnervation procedures on the rabbit rectus femoris muscle (using a saphenous nerve graft connected to motor nerve to vastus medialis) was undertaken by Rab *et al.* [14]. Muscles were assessed physiologically and histologically 15 months following reinnervation with a 7cm nerve graft coapted to the muscle's motor nerve either during a single operation or via 2 stages separated by 8 months. Physiologically the muscles subject to a 2 stage procedure demonstrated a significantly improved outcome compared to those undergoing a single stage. Similarly the histomorphological profiles of the muscles demonstrated that whilst both groups had mean fibre diameters of less than those of the control muscles, the 2 stage group, which had the better physiological outcome measurements, had an increased mean Type IIb (fast twitch glycolytic) fibre area. This finding led the authors to conclude that "morphological analysis of the reinnervated muscle tissue also allows us to interpret the functional outcome of the grafting procedures in groups 1 and 2." It is also of note in this study that the motor nerve to which the graft was coapted was that of the neighbouring vastus medialis muscle, and at 15 months post procedure fibre type composition was the same as that seen in the vastus medialis muscle itself. On the basis of the findings of the aforementioned studies by Frey *et al.* and Rab *et al.* that the idea of direct correlation between muscle histomorphology and functional outcome post surgical transfer was established.

The normal fibre type composition of the rectus femoris muscle in the New Zealand White rabbit has been detailed in previous studies [11] [12] [15]. Typical values for fibre

type composition are given in Table 6.5.1.1. It can be seen from the data that there are some striking differences between the findings from the current experiment (the control rectus femoris muscle fibre type composition values) and those detailed in Table 6.5.1.1.

Typical muscle fibre type counts varied between all of the aforementioned studies, however exact detailing of the methodology used was absent in the case of the work of Frey [12]. Examination of previous work published by the same author revealed that entire muscle cross sections were counted totalling between 1700 and 3000 individual fibres in each case, with the assumption being that this was the case in his 1998 work [11] [16] [17]. Rab *et al.* described examining an average of 300 muscle fibres in 3 randomly chosen fields from serial sections of the entire muscle. Again, no total muscle fibre count number was given, leaving the reader to question whether 300 fibres were counted in each section (10μm sections in a muscle averaging 8cm in length cut longitudinally in half, would equate to roughly 480000 muscle fibres per muscle) or if 900 fibres were counted in total (300 fibres x 3 fields – a much more likely figure) [15]. Yamada analysed 300 individual muscle fibres, taken to be representative of the muscle as a whole, with Guelinckx analysing 150 fibres, both taking their samples from standardised muscle sections [18] [19]. The use of a standardised muscle section together with the use of automated image analysis software was undertaken by Sterne *et al.* in their study looking at nerve regeneration and its effects on the target organ (muscle) [20]. In this study 900 individual muscle fibres were examined typed and sized, with particular reference to fibre type grouping also being undertaken. Additionally in a further study using the rectus femoris muscle model in the rabbit Jamali *et al.* used a total fibre count of 450 individual muscle fibres spread over 3 muscle sections (with approximately 9 fields examined per section) to be representative of the muscle as a whole when looking at muscle fibre innervation status using NCAM as a marker [21].

Taking all of the above into account and examining the previously published data given in Table 6.5.1.1, it can be seen that similar values for fibre type distribution were achieved in each case, using a similar or smaller number of individually counted muscle fibres as was used in this study (excepting the assumptions made for Frey *et al.*). This supports our initial methodological decision (reached after examining all of the aforementioned investigations' methods) to take a standard mid-muscle section with 4 random fields, giving a total fibre count of approximately 300, as representative of the

muscle as a whole. This being the case the disparity seen between the percentage of type IIa fibres observed in our control muscle results and those detailed in the other studies must be due to something other than insufficient sampling[2].

Table 6.5.1.1: Rabbit rectus femoris muscle fibre type composition values expressed as a percentage of total fibre counts, according to published literature.

Fibre Type	Frey[3]	Guelinckx[4]	Yamada[5]	Rab[6]	MacQuillan
I	7.5%	3%	8.8%	8.4%	4.8%
IIa	25.4%	25%	-	20.3%	1.6%
Iib	67.1%	73%	91.2%	71.3%	93.6

The common thread between the studies detailed previously in the text and referred to in Table 6.5.1.1 is that in all cases the fibre typing was undertaken using enzymatic techniques. The drawback with such techniques is that the results are very much

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[2] The practice of using multiple fields within a whole sample, taken to be representative of the sample as a whole, are borne out in the methodology of the discussed papers and is seen in numerous other areas of bioscience publication, it is commonly known as "statistical inference". Geuna, S., D. Gigo-Benato, and A. De Castro Rodrigues, *On sampling and sampling errors in histomorphometry of peripheral nerve fibres.* Microsurgery, 2003: p. 72-76.

[3] 12. Frey, M., P. Giovanoli, and C. Meuli-Simmen, *Quantification of different free muscle transplants to reconstruct mimic function: an experimental study in rabbits.* Plastic and Reconstructive Surgery, 1998. **101**(7): p. 1774-1783.

[4] 19. Guelinckx, P.J. and J.A. Faulkner, *Parallel fibred muscle transplanted with neurovascular repair into bipennate muscle sites in rabbits.* Plastic and Reconstructive Surgery.1992. **89**(2): p. 290-298.

[5] 18. Yamada, A. and K. Harii. *Experimental study on free muscle trnasplantation with neurovascular anastomosis in rabbits - Discussion.* in *2nd Vienna Muscle Symposium.* 1985. Vienna: Facultas.. In this study muscle fibre type differentiation was limited to type I and type II fibres only.

[6] 15. Rab, M., et al., *Histomorphology of rabbit thigh muscles: establishment of standard control values.* J Anat, 2000. **196**: p. 203-209.

dependant on incubation at exact pH's to achieve correct fibre type differentiation, the preparations are highly temperature sensitive, the method requires multiple slides to produce one set of data and the preparations deteriorate with time [23].

Recent advances in immunohistochemical techniques have allowed the use of monoclonal antibodies to identify the differing isoforms of myosin (which equate with the isoforms of myofibrillar ATPase) and to differentiate between muscle fibre types on both frozen and paraffin muscle sections. This technique has the advantages of being easily reproducible and yielding a permanent result, and it has been shown to produce equivalent results to that of enzyme studies [23]. Due to the inherent advantages of being able to differentiate between all 3 fibre types studied on a single slide and the fact that preparations were permanent this method of fibre typing was chosen for the current body of work.

As stated previously the conceptual basis for muscle fibre typing is that of a group of muscle fibres with reasonably similar functional properties. Thus separation should ideally be based on purely functional criteria. However for practical reasons the distinction between fibre types has come to rest on histochemical differences: this method of classification has allowed the scientific community to replace the old concept of red and white muscle fibres [24]. Several enzymatic methods have been used for the classification of muscle fibre type almost interchangeably [25] [26] [27], however it has been definitively demonstrated that differing protocols produce differing results and that indeed there are more than just 2 sub-groups of type II fibres[7], with differing preparations assigning these additional groups to IIa or IIb in a varying fashion [1]. This has led to some authors to comment "Despite several reports of lack of correspondence between different classification methods, investigators continue to use the same designations for groups of muscle fibres separated by different techniques. This is more than unfortunate

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[7] Up to 4 different myosin types have been identified using immunohistochemical methods, with up to 8 types being reported using enzymatic techniques . Romanul, F.C.A., *Enzymes in muscle I. Histochemical studies of enzymes in individual fibres.* Arch Neurol, 1964. **11**: p. 355-368. Gorza, L., *Identification of a novel type 2 fibre population in mammalian skeletal muscle by combined use of histochemical myosin ATPase and anti-myosin monoclonal antibodies.* J Histochem Cytochem, 1990. **38**: p. 257-265..

and it makes it difficult or even impossible to compare results from different laboratories" [1].

The disparity between our control rectus femoris muscle fibre typing results and those previously published may well be explained by the aforementioned facts. The monoclonal antibody used to define type IIa fibres in this study could have exclusively labelled these fibres leaving all the "intermediate" type II fibres[8] to be bundled in with the type IIb fibres. The reverse of this phenomenon occurring in enzymatic preparations would explain the disparities, as without doubt significant and easily defined populations of type IIa fibres were identifiable in our tissue preparations. Another possibility is genetic variability within the rabbit population (our rabbits coming from a different source from those used in the Austrian series[9]).

As with previous studies the results seen in the muscle transfers demonstrated fibre type grouping in the muscle sections examined, not apparent in the control sections. Additionally further changes in the fibre type composition and size of the myocytes reflected those reported previously. At both time points in both of the peripheral reconstruction groupings, type I fibre composition decreased, as demonstrated in figure 6.4.1. (although there is some overlap between the 95% confidence intervals of the control and 9vl groups). These findings tie in with the physiology outcomes for the groups (ie: there is no difference between the groups, seen again in figure 6.4.1 with overlap between the confidence intervals of all experimental groups). Type IIa fibre percentage results did not show any differences between groups, nor between muscle transfers and the controls. Figure 6.4.2 demonstrates this with overlap being seen with the confidence intervals of all groups and the controls. It can also be seen that the data for the 6vl group displays an unusually wide 95% confidence interval when compared to the others although the reason for this is unclear. The data for the 9vl group is in keeping with both rf groups and the data spread within the 6vl group does not contain single values responsible for a skew, but contains 2 flaps without IIa fibre content and 4 tightly clustered above the mean. Data for the type IIb fibre percentages is given in figure 6.4.3. As a result of the anomalous IIa fibre type findings in the 6vl group, this again leads to

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[8] These have been assigned various designations in the literature including IId and IIx.

[9] The works of Rab *et al.* and Frey *et al.*.

the large confidence intervals seen in this group for type IIb fibre counts. It is evident that both the rectus femoris groups have a significantly increased proportion of type IIb fibres than that of the controls, a reflection of the previous fibre type findings discussed above. It is hard to draw conclusions about the 6vl group for the reasons already outlined, though the 9vl group appears to have a type IIb fibre composition somewhere between the rf groups and that of the controls. It is difficult to interpret this last result as there is little readily identifiable published data on the fibre type composition of the normal vastus lateralis muscle, and that this lower percentage population of type IIb fibres in the vl groups (compared to the transferred rf muscles) may be a function of a different fibre type distribution within the normal vl muscle, or it may simply be attributable to sampling and with larger group sizes the vl transfers may have attained a profile similar to the rf transfers. It is unlikely however that such small percentage changes in fibre type composition would have physiological significance, a view that has previously been expressed by other authors [30].

No significant differences were noted between any of the operated groups or the control group in terms of fibre cross sectional area when both type I and IIa muscle fibres were examined (figures 6.4.4 and 6.4.5). Mean fibre cross sectional area for the type IIb fibres (the main fibre type) is shown in figure 6.4.6. Once again there is overlap of the confidence intervals between the muscle transfer groups, and individually with the control group (excepting the 9rf group). All the muscle transfers do however have lower mean values for mean fibre cross sectional area (on average between the groups as a whole 68% of that of the control group). This figure is similar to the values seen in similar experiments undertaken by Guelinkx *et al.* who demonstrated mean fibre cross sectional area of 63% of that of the controls in 2 sets of rectus femoris muscle transfers, and slightly higher than that of Frey *et al.* who reported a mean cross sectional area of 51% of that at control fibres [30] [19] [11]. The similarity of transfer cross section area is again in agreement with physiology findings.

Overall the morphological appearance of the muscle transfers, both at 6 and 9 months post operatively, demonstrate a high degree of concordance between the operated groups and this is reflected in the similarity of the physiological profiles of the groupings. Additionally our results are in agreement with the findings of the majority of experimental

work that has been undertaken on free functional muscle transfers, noting decreases in the percentage of type I muscle fibres and a 30 - 40% reduction in mean fibre area.

6.5.2 Facial Reanimation Model

Morphological characteristics of the mimic musculature in the human are known to differ somewhat from that of typical striated peripheral musculature. Mean muscle fibre diameter is reported as approximately 50% of that seen in human limb musculature and type IIb fibres are more scanty than in their peripheral counterparts[10] [31]. In the rabbit, varying values for mean mimic muscle fibre diameter have been reported, with facial muscle fibre size ranging from 58 – 81% of that of the peripheral musculature diameters [18] [12]. Additionally mimic muscles in the rabbit have a fibre type profile typically consisting of 20 – 46% type I fibres and 20 – 50% type IIb fibres [12] [18] [7]. Details of values given in the literature are shown in table 6.5.2.1.

Previous studies have demonstrated that the facial nerve has the ability to alter muscle fibre type composition, when the mimetic muscles of the face of the rabbit have been the subject of cross reinnervation [7]. Similarly when peripheral limb muscles (such as the rectus femoris or the pectoralis descendens) have been coapted to the facial nerve as a free functional muscle transfer, alterations in muscle fibre type profile have also been reported [18] [13] [12].

The fibre type distribution of the rectus femoris muscle is detailed in section 6.5.1 together with a discussion regarding the control muscle values seen in this experiment (tables 6.4.1 and 6.4.2). When compared to the control muscle data, the mean percentage of type I fibres is increased in all operated groups (figure 6.5.7). With the exception of the 6bb group the 95% confidence intervals for the operated groups show minimal or no overlap with those of the control muscle group. In the case of the former

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[10] Type IIb fibres are replaced by type IIc fibres, extremely scant in limb muscles and often regarded as pathological, though the authors admit that enzyme technique differences may be the cause of this finding (see Section 6.5.1).

(6bb), the large range in the confidence intervals is likely to be reduced if a bigger sample were undertaken. The mean value for the 6bb group is in keeping with the rest of the operated groups and it is entirely possible that if re-sampling or a larger sample size were used, a real difference between this group and the controls would become apparent. All operated groups demonstrated similar mean values for type I muscle fibre composition with considerable overlap of confidence intervals.

Table 6.5.2.1: Rabbit mimic muscle data relating to fibre type composition and fibre diameter. Figures given for the fibre types denote percentage composition of the muscle and mean fibre diameter.

Study	Muscle	Type I	Type IIa	Type IIb
Frey[11]	scutuloauricularis	41.8% / 41.4µm	37.4% / 43.9 µm	20.8% / 50.6 µm
N – Tairych[12]	scutuloauricularis	32.7% / 43.5 µm	14.2% / 51.4 µm	53.1% / 53.1 µm
N – Tairych	buccinator	46.1% / 45.2 µm	14.8% / 51.4 µm	39.1% / 52.6 µm
Yamada[13]	facial	23% / -	- / -	77% / 34 µm

Mean type IIa fibre percentage composition was increased over the control mean in all operated groups, with a wide confidence span again being noted in the 6bb group. Whilst the same argument applies to the latter, as in the case of the type I fibre distributions, an unexpected result was seen in the case of the 9mm group which demonstrated a similar mean value and confidence interval spread as that of the control group. A low intra group variation was also noted, leading to the possibility that this was

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[11] 12. Frey, M., P. Giovanoli, and C. Meuli-Simmen, *Quantification of different free muscle transplants to reconstruct mimic function: an experimental study in rabbits.* Plastic and Reconstructive Surgery, 1998. **101**(7): p. 1774-1783.

[12] 7. Nehrer-Tairych, G.V., et al., *The influence of the donor nerve on the function and morphology of a mimic muscle after cross innervation: an experimental study in rabbits.* British Journal of Plastic Surgery, 2000. **53**: p. 669-675.

[13] 18. Yamada, A. and K. Harii. *Experimental study on free muscle trnasplantation with neurovascular anastomosis in rabbits - Discussion.* in *2nd Vienna Muscle Symposium.* 1985. Vienna: Facultas.. In this study no sub–typing of type II fibres was undertaken and an overall mean fibre diameter was given. No specific details of the muscle examined is given in he text, the tissue simply being referred to as "facial muscle".

indeed an accurate reflection of the 9mm population. This finding is hard to explain, as it is not in keeping with the other operated groups though the confidence intervals do overlap, albeit barely, as can be seen in figure 6.4.8. It is however unlikely that this difference between operated groups is responsible for the physiological differences noted between the mm and bb groups at the 9 month time point, especially given that the 9mm group demonstrated the highest proportion of type IIb fibres (known to have the highest tetanic force output of all 3 fibre types) out of all operated groups (figure 6.5.9). Once again the 6bb group displayed a mean fibre type percentage in keeping with the rest of the operated averages together with a variance approximately twice the size of any of the other operated series. Aside from the 6bb data, the confidence interval overlaps between operated and control groupings was minimal, emphasizing the ability of the facial nerve to re-specify muscle fibre type.

No significant differences were noted between any of the operated groups or the control group in terms of fibre cross sectional area when both type I and IIa muscle fibres were examined (figures 6.4.10 and 6.4.11). Type IIb fibre cross sectional area was decreased in all of the operated series compared to the controls as seen in figure 6.4.12. The muscle transfers reinnervated by the larger nerve (6bb and 9bb) showed overlap between their 95% confidence intervals and that of the control series. All transferred muscle groups had mean area values of approximately half of that of the control, with no difference discernable between them (as demonstrated by the fact that an almost complete overlap of confidence intervals in the operated groups was seen). Although reinnervation of the rectus femoris muscle transfers using the smaller of the cranial nerves resulted in a significant difference in fibre size compared to the controls this could not explain any physiological differences noted since no difference in resultant reinnervated muscle fibre area was seen when large and small nerves were compared.

Looking at these results as a whole, once again a high degree of concordance was noted between groups both in terms of muscle fibre type composition and mean fibre area. The fibre type profiles from all operated groups is, within the limits discussed above, sufficiently different from the control group, that it can be stated that coaptation to the facial nerve has resulted in muscle fibre type re-specification to more closely match that of the native facial musculature. Comparison of rectus femoris muscle fibre type composition post transfer with that of the native facial musculature is difficult, as no facial

muscle biopsies were undertaken in this study, and although comparing results from previously published work is not entirely satisfactory, for reasons discussed earlier in the text (section 6.5.1), some information can be gained. Table 6.5.2.1 summarises the results of the work of Frey and Yamada, the 2 published series whose work most closely parallels this current study.

Table 6.5.2.2: Published data on rabbit mimetic muscle composition and functional muscle transfers undertaken coapted to branches of the facial nerve. All figures are given in the format of type I / IIa / IIb fibres with the units in brackets following. Unavailable data is represented by (-) annotation.

Study	Frey[14]	Yamada[15]	Current
Donor nerve	n. scutuloauricularis	buccal	combined[16]
Donor muscle	scutuloauricularis	facial	-[17]
Fibre type profile	41.8 / 37.4 / 20.8 (%)	23 / - / 77 (%)	-
Fibre diameter	41.5 / 43.9 / 50.6 (µm)	- / - / 34 (µm)	-
Transferred muscle	rectus femoris	rectus femoris	rectus femoris
Fibre type profile	7.5 / 25.4 / 67.1 (%)	8.8 / - / 91.2 (%)	4.7 / 1.5 / 93.6 (%)
Post transfer	17.2 / 21.2 / 58.1 (%)	28 / - / 72 (%)	14.2 / 5.0 / 80.8 (%)
Fibre diameter	66.8 / 54.7 / 62.1(µm)	- / - / 58 (µm)	36.0 / 36.2 / 48.0(µm)
Post transfer	60.4 / 54.1 / 58.1(µm)	- / - / 47 (µm)	29.3 / 33.5 / 33.7(µm)

In all 3 sets of results a decrease in the proportion of type IIb fibres is reflected by a similar increase in the type I fibre population, with the IIa fibre ratios remaining comparatively stable (discrepancies between rectus femoris type IIa fibre populations

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[14] 12. Frey, M., P. Giovanoli, and C. Meuli-Simmen, *Quantification of different free muscle transplants to reconstruct mimic function: an experimental study in rabbits.* Plastic and Reconstructive Surgery, 1998. **101**(7): p. 1774-1783.

[15] 18. Yamada, A. and K. Harii. *Experimental study on free muscle trnasplantation with neurovascular anastomosis in rabbits - Discussion.* in *2nd Vienna Muscle Symposium.* 1985. Vienna: Facultas.

[16] Figures quoted for this study are pooled values for both the mm and bb groups as no differences in fibre type profiling was seen inter group, therefore the donor nerves are both the buccal and marginal mandibular branches of the facial nerve.

[17] As stated in the text no facial muscle biopsies were undertaken in this experiment.

have been addressed earlier in the text). In addition to this all muscle transfers underwent a degree of fibre atrophy. The overall mean decrease in fibre size in the operated groups from this experiment was 19% post operatively, a figure matching that found by Yamada. Frey *et al.* noted a 6.2% decrease in mean fibre size in their study, however unlike this experiment and that of Yamada only a small portion of the whole rectus femoris muscle was transferred to the face with its inset being entirely different. It is likely that the workload imposed on the muscle transfer was different in Frey's study and this probably accounts for the lower degree of muscle atrophy experience by the fibres in this instance. In neither the case of the work of Frey or Yamada did the transferred muscle undergo fibre atrophy resulting in fibre diameters equivalent to that of the native mimic musculature. This leads to the conclusion that whilst the facial nerve is able to respecify the muscle fibre type of transferred peripheral muscle, the muscle retains fibre sizing features more akin to its original configuration than that of the native mimic musculature the facial nerve normally innervates.

Overall, the operated groups display remarkable homogeneity with respect to muscle fibre typing and size. The results agree broadly with other published work on the subject, as far as valid comparisons can be made. With respect to the differences that are apparent between the groups in terms of physiological outcome (Chapter 3) they cannot be attributed to variations in the fibre typing specificites of the nerves used to reinnervate the muscle transfers (ie; it would appear that the marginal mandibular nerve is equivalent to the ventral ramus of the buccal branch of the facial nerve in all aspects excepting absolute axonal numbers). This concurs with the results of the previous chapters, and lends weight to the hypothesis that it is the absolute axonal number used for reinnervation purposes that is the important factor when determining (functional) outcome in functional muscle transfers coapted to the facial nerve.

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Chapter 7

Conclusions

7.1 Conclusions

The results of the current series of experiments reported in this body of work were examined in order to ascertain if the hypotheses of this project could be validated or discounted.

7.1.1 Hypothesis

The variability of results witnessed clinically following facial reanimation surgery is due to differing axonal loads being used to reinnervate a (relatively) constant sized muscle mass. Too few axons reinnervating the muscle transfer results in recruitment of insufficient muscle fibres and poor post operative movement, whilst the recruitment of the optimum number of muscle fibres (so as to balance the force of pull of the normal contralateral musculature) by the optimum number of axons (not necessarily the most number of axons) will produce dynamic symmetry as the end result. The phenomenon of late onset "tightening" described in the literature [1] is the result of over stimulation of the muscle transfer as a result of the recruitment of too many muscle fibres by an inappropriately large reinnervating axonal load.

Peripheral limb reconstruction using functional muscle transfers produce disappointing results in terms of force production when compared to the intact contralateral musculature, the best results quoting a maximum of 50% force recovery in the reconstructed site [2] [3]. It is thought that incomplete reinnervation of the muscle is responsible for this either due to an insufficient reinnervating axonal load (the recipient nerve being too small to fully reinnervate the muscle transfer) or due to axonal escape at the site of neurorrhaphy robbing the muscle of motor axons. By improving the odds of muscle fibre reinnervation by increasing the reinnervating axonal load improved muscle functional ability should be achieved.

The experiments undertaken in this current work were designed so as to approximate the clinical scenarios described above as closely as possible, thereby testing the hypothesis.

7.1.2 Physiology

Examining the physiology results in the peripheral reconstruction setting, several features are apparent from the presented data. The muscle transfers undertaken in this series of experiments demonstrated tetanic force production to be between 60% and 80% of that of the contralateral rectus femoris muscle. These results demonstrate a high concordance with figures published by other groups undertaking similar work in the same field [4] [5] (this therefore attests to the fact that inadequate surgical technique undertaken in this work is not responsible for the differences, or lack thereof, in outcomes seen). Looking specifically at differences seen between the large and small reinnervating peripheral nerve groups, it is readily apparent the use of a large (double size) nerve confers no advantage over that of the indigenous (small) nerve. It is also apparent that regardless of the size of nerve used to reinnervate the muscle, complete reinnervation had occurred by the 6 month time point (demonstrated by the fact that no further improvements in force generation were seen beyond this point).

Considering the facial reanimation model data series, differences between the reinnervating characteristics of peripheral and cranial nerves become apparent. It has been demonstrated that significant improvements in physiological performance can be gained in the muscle transfers by the use of a large reinnervating axonal mass compared to a small one. Furthermore it is also appreciable that the large nerve continues to recruit muscle cells up to the 9 month time point, where as the small nerve fails to achieve any significant improvement beyond 6 months. It is known from previously published experimental work that the ability of cranial nerves to expand the size of their motor units is relatively limited compared to peripheral axons (roughly speaking 2:1 versus 5:1 respectively) [6]. The outcomes seen in the facial reanimation groups support this, as functional recovery of the transfers was well below the 50% mark suggesting failure of complete muscle reinnervation. This also sits neatly with other published data that demonstrated 90% recovery of function in orthotopic transfer of the scutuloauriculatis muscle in the rabbit [7] – whilst this may be eminently achievable in small muscles reinnervated by their indigenous nerve, the muscle transfers in this series failed to fully reinnervate due to inability of the cranial nerves used to expand their motor unit size sufficiently.

The phenomenon of continued recruitment of muscle fibres by the larger nerve beyond six months probably has its origins here in limited motor unit expansion; the only other work that undertook whole muscle transfer of the rectus femoris muscle to the cheek used a constant sized nerve (a branch of the buccal division of the VII nerve) [8]. That study too failed to note any improvement in outcome beyond 5 months post operatively, with results measured up to the 12 month time point. It seems likely that these transfers underwent a similar post operative course to the marginal mandibular groups in this study, with initial reinnervation being undertaken in the normal fashion with axons being guided down the distal nerves sheaths (the degenerating bands of Bugner, distal to the neurorrhaphy), to the site of the previous motor end plate, and re-establishing the neuromuscular junction. Neighbouring myocytes still devoid of axonal input would be recruited by motor unit expansion until the limit of the nerve is reached in terms of reinnervation potential. This may well occur before the 6 month post operative time point but it can only be stated for certain that it has occurred by the 6 month time point, and indeed this is also the case in the peripheral groups. It is speculated that the likely reason for the continued recruitment of muscle fibres seen in the large nerve group is that when axons are unable to grow directionally down a distal nerve sheath they, are required to find their own "indirect" route to the target organ and then establish a new neuromuscular junction, from where motor unit expansion may then also occur. In essence the majority of the distal nerve sheathes may be occupied by the large nerve group at the 6 month time point, however due to fewer nerve fibres only a minority are occupied in the small nerve. In both cases the reinnervating axons would be expected to have recruited their full complement of muscle fibres. The larger nerve having recruited more myocytes therefore produces an improved functional outcome, as demonstrated in the experimental results. The smaller nerve however has no more axons to fill the remaining empty distal nerve sheathes, while the converse is true for the larger nerve and further reinnervation of the muscle transfer occurs at a slower pace as those axons growing outside a band of Bugner make their own way towards the target – hence the improved functional outcome seen in the large nerve group between the 6 and 9 month time points.

7.1.3 Nerve Histomorphometry

Considering the nerve histomorphology results in the peripheral setting the first observation made is that the neuronal profiles are stable within each group between the 6 and 9 month time points. This leads to the conclusion (already alluded to in section 7.1.2) that full reinnervation has occurred by 6 months and that the pruning phase of axonal filaments has taken place. The smaller axonal fraction crossing the neurorrhaphy in the large nerve group signifies pruning or axonal die back has taken place in the face of axons failing to establish connection with a target. This in turn implies that the muscle is refractory to further reinnervation and that any myocytes that had been receptive to reinnervation have had the neuromuscular connection re-established. In short, adequate reinnervation of all receptive myocytes is achieved without the need of an oversized nerve. Failure to produce complete functional recovery probably lies in the inappropriate sensory neurotization of myocytes by sensory afferent fibres, leading to a non functional population of muscle fibres refractory to motor axon neurotization. The high proportion of sensory afferent fibres in the peripheral motor nerves (roughly speaking a 60:40 split motor:sensory) makes it unlikely that simply increasing the absolute number of axons will improve outcomes, as the proportions do not change. It would appear from the literature however that when sufficiently large numbers of axons are used to reinnervate muscles (increased by factors of 5 or greater), then advantages conferred on the motor axons to reinnervate myocytes become evident [9] [10]. Inappropriate sensory neurotization is further endorsed by studies that have reported up to 90% muscle force recovery for mimetic muscle transfers reinnervated by branches of the facial nerve [7]. It is known that sensory afferent fibres for the mimetic musculature run mostly in the V nerve, thereby markedly reducing the possibility of sensory neurotization of myocytes, perhaps accounting for the superior results seen when comparing mimetic muscle orthotopic transfers to those undertaken in the periphery.

Improved axonal number and total cross sectional area distal to the site of neurorrhaphy was noted at both time points for the large nerve in the facial reanimation groups. This demonstrates concordance with the physiology results. However the relationship between these indices and muscle force production is not linear and difficult to define precisely, though bluntly speaking increased axonal number and total cross sectional area equates to improved functional outcome. In reference to the hypothesis of over stimulation of muscle transfers being responsible for late onset "tightening" of the

muscles that has been reported, the findings of the large nerve group may provide a clue as to one possibility. Reinnervation of the proprioceptive components of muscles is known to be poor (at best) following division of the motor nerve to the muscle, and so transfers to the face are likely to be largely devoid of such input [11]. The situation of increasing neural input into the muscle is one that equates with improved functional ability of the aforementioned organ, but one in which there is no proprioceptive feedback – a similar situation in the periphery where proprioceptive modulation is interrupted leads to spasticity. Up to a point the contra lateral musculature of the functioning hemi face, soft tissue inertia and elasticity of the ipsi lateral side will act against the muscle transfer, preventing the tendency of increased resting tone to result in deviation (or tightening) towards to the side of the transfer. However, once a critical mass of reinnervated muscle fibres is reached they will overcome such forces resulting in the tightening described. The continued recruitment of muscle fibres by the reinnervation process described above would account for this late onset phenomenon.

7.1.4 Intramuscular Immunohistochemical Studies

Intramuscular neural regrowth, a direct measure of muscle reinnervation, was assessed using PGP 9.5. The peripheral limb reconstruction model groupings demonstrated no differences between big and small reinnervating axonal loads at either time point, echoing the findings of both of the other aforementioned outcome measures. With the facial reanimation groupings when examining the pattern of intramuscular neural regrowth or the "reinnervation index" (see Chapter 5 section 5.4.1), it can be seen that the number of axons in the segment of motor neurone distal to the neurorrhaphy predicts the amount of neural regrowth witnessed within the muscle. At both the 6 and 9 month time points the large nerve had significantly improved reinnervation indices when compared to the small. There was good correlation noted between PGP 9.5 results and both physiology and nerve histomorphometry results. It can therefore be concluded that PGP 9.5 is a useful marker in determining the extent of intramuscular neural regrowth or reinnervation. The other immunohistochemical technique employed to provide a direct measure of muscle reinnervation (as opposed to the indirect means of assessment using muscle physiology and nerve histomorphometry) was NCAM. This marker, present only in denervated adult mammalian muscle, demonstrated correlation with the PGP 9.5 data for the peripheral limb reconstruction groupings, with a significantly higher proportion of

denervated muscle fibres in the marginal mandibular nerve group compared to the buccal branch group in the facial reanimation model at the 9 month time point. These results however, were not directly comparable to either the physiology or nerve histomorphometry data. Previous published studies have also commented on difficulties in correlating NCAM findings with the physiological performance of a muscle, and this agrees with the findings presented in this current work [12]. Several reasons may contribute to this including the fact that the data is non parametric, but it may also be that the marker itself is not sensitive enough. Another possibility is that the majority of muscle fibres failing to attract neural input have undergone degeneration by the time specimens were examined at 6 months, with only a residual population of denervated fibres, refractory to degeneration remaining at this stage resulting in little difference between specimens. The calculation of NCAM populations as a percentage area of total endomyceal area rather than of total muscle fibres may offer one possible solution and should be considered when undertaking future work. Overall however, the use of NCAM, given the findings reported by this study, as a qualitative marker of muscle reinnervation would seem limited to demonstrating that reinnervation had taken place by virtue of the fact that the overwhelming majority of intact muscle fibres failed to express it.

7.1.5 Muscle Immunohistochemistry

Muscle histomorphometry studies using immunohistochemical techniques to identify type I, IIa and IIb muscle fibres produced differing results for type IIa fibre content in the control groups when compared to previously published enzymatic staining techniques [7] [13]. Within the peripheral reconstruction groupings there was an overall decrease in the type I fibre population, and an increase in the type IIb population (the type IIa population remaining static) following reinnervation, however it is improbable that the small percentage changes seen are likely to impact on physiological outcome. An average decrease in mean fibre cross sectional area of 30% when compared to controls would be a more plausible reason for the difference in physiological performance of the muscle transfers. High concordance between the fibre content and size in both groups at both time points agrees with the other outcome measures reported, and such changes also agree with the published literature [4]. Greater degrees of muscle fibre composition change were noted in the facial reanimation groups, with an overall increase in type I and IIa fibre percentages at the expense of type IIb fibre number. This confirms the

ability of the facial nerve to reassign muscle fibre type of peripheral skeletal muscle to that more closely matching fibre type composition of mimetic musculature. There were no significant differences in fibre type composition between the groups, with a decrease in fibre size noted to a similar extent in all groupings. This points to the fact that neither muscle fibre type composition or size is responsible for physiological differences noted in the facial reanimation groupings, and that it is the number of reinnervated fibres alone that is likely to be responsible for the improved outcome noted in the large nerve group. In this respect these findings agree with those of the physiology and nerve histomorphometry sections.

7.2 Overall Conclusion

In conclusion therefore following the series of experiments detailed in this thesis, the evidence presented and discussed supports the hypothesis that the use of a larger nerve to reinnervate a functional muscle transfer will improve outcome in terms of physiological performance when done in the setting of the central nervous system (and that this may account for the phenomenon of late on set tightening due to reinnervation characteristics of the larger nerve). In the peripheral nervous system the use of an increased axonal load above and beyond that normally supplied to the muscle confers no added benefit in the field of functional muscle transfer.

7.3 Surgical Implications

In the peripheral limb reconstruction setting the findings of this work suggest that using an oversized nerve (when compared to that normally used to innervate the muscle that is to be used as the functional transfer) has no added benefit but could result in increased morbidity at the recipient site. By matching the reinnervating nerve size to that of the indigenous motor nerve to the muscle used in the transfer, it may be possible to achieve force production of 60% to 80% of the contralateral muscle the transfer aims to replace assuming that the muscles are matched for size and type.

Considering facial reanimation surgery, the results of this work demonstrates that differing force outputs are achieved from a constant sized muscle transfer when the reinnervating axonal load is varied. This suggests the possibility of tailoring the muscle

transfer to achieve the optimum outcome by varying the axonal input. If it were possible to define the optimum number of reinnervating motor axons required to produce dynamic symmetry of the face following facial reanimation surgery (for example by undertaking a large series biopsy of the cross facial nerve graft being coapted to a constant sized muscle transfer[1] and matching axonal input with surgical outcome) then preoperative biopsy and analysis of the end of the reinnervating motor nerve (graft) would allow surgeons to include or exclude the required number of fascicles to produce the best result for the particular muscle transfer. An alternative approach would be to aim for full reinnervation of smaller sized muscle transfers, almost equivalent in size to that of the native facial musculature, as undoubtedly the larger muscle transfers currently favoured do not achieve anything like full reinnervation status due to the inability of the cranial nerves to expand motor unit size sufficiently. This would also require further work to determine the motor unit sizes of the proposed donor branches of the facial nerve, and the muscle fibre counts of potential donor muscles. Additionally force production data for the native facial musculature would need to be calculated so that muscle transfers could be balanced against this.

By tailoring the axonal input into muscle transfers it may be possible in the future to produce balanced dynamic motion as the result of all functional muscle transfers used in facial reanimation and additionally avoid the phenomenon of late onset tightening.

7.4 Future Directions

Continued research into the field of facial reanimation has avenues both in the clinical and experimental settings. The direction of clinical research would be well employed in the manner alluded to above (Chapter 7 section 7.3). Further experimental work aims to examine the effects of differing sized nerve grafts on reinnervation of a constant sized muscle transfer coapted to a constant sized donor nerve. The proposed idea is that the graft could be used in a similar fashion to a rheostat when controlling electricity current (by limiting the number of motor axons that can grow down it), and that if this is the case

[1] This would require a single muscle to be used in the reanimation series, such as pectoralis minor which does not require trimming prior to inset and so provides a relatively constant sized muscle similar to the rectus femoris used in this series of experiments.

the number of fascicles used in a cross facial nerve graft could be used to limit the number of motor axons reinnervating the muscle transfer (averting over stimulation of the muscle transfer) thereby delivering a reproducible number of motor axons at the distal end of the cross facial nerve graft no matter how large the donor branch of the facial nerve.

Future work within the field of peripheral limb reconstruction using functional muscle transfers should be directed towards defining the minimum number of motor axons that can be used to achieve complete reinnervation of the muscle transfer. It should also investigate methods of improving functional recovery from the ranges reported by this and other studies (60% to 80% recovery) to figures in the region of 95% plus. Potential areas of interest may involve the development of means to avoid inappropriate sensory neurotization of muscle fibres and to improve proprioceptive function of the muscle post transfer.

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Appendix 1 - Stimulation protocol

Stimulation protocol carried out in sequence from top down for each muscle (both transplants and controls).

Stimulation frequency	Pulse duration	Voltage	Period of stimulation	Interstimulatory gap
100Hz	0.2ms	10V	1s	10s
100Hz	0.2ms	10V	1s	10s
100Hz	0.2ms	10V	1s	10s
10Hz	0.2ms	10V	1s	10s
20Hz	0.2ms	10V	1s	10s
30Hz	0.2ms	10V	1s	10s
40Hz	0.2ms	10V	1s	10s
50Hz	0.2ms	10V	1s	10s
60Hz	0.2ms	10V	1s	10s
70Hz	0.2ms	10V	1s	10s
80Hz	0.2ms	10V	1s	10s
90Hz	0.2ms	10V	1s	10s
100Hz†	0.2ms	10V	5s	10s

† Force fatigue rate measurement.

Appendix 2 - Nerve Processing

Preparation of PIPES buffered 2.5% gluteraldehyde solution

Reagents	
PIPES (piperazine-N-N'-bis(2-ethane sulphonic acid))	3.024g
1M NaOH	18ml
Distilled water	to100ml
10% purified gluteraldehyde	

Method

To make PIPES buffer: add solid to approximately half the required volume of water, add the NaOH and stir to dissolve. Make up to 100ml in either a volumetric flask or a measuring cylinder. Adjust pH to 7.6 (keep in refrigerator). To make fixative: add 2.5ml 10% gluteraldehyde and 7.5ml PIPES buffer, mix well (store in refridgerator).

Preparation of osmicating solutions

PIPES buffered 2%OsO_4 + 3%$K_3Fe(CN)_6.3H_20$	
OsO_4	2g
$K_3Fe(CN)_6.3H_20$	3g
PIPES buffer	to 100ml
PIPES buffered 6% $NaIO_3$ + 4% sucrose	
$NaIO_3$	6g
Sucrose	4g
PIPES buffer	to 100ml

Preparation of epoxy resin

Reagents	
Epoxy resin (Durcupan A)	10ml
Dodecenyl succinic anhydrate (DDSA) (Durcupan B)	10ml
Di-*n*-butyl phtalate	0.25ml
Methyl nadic anhydrate	0.2ml
BDMA	0.5ml

Method

Measure resin and DDSA into a disposable container using syringes and stir well with a glass rod. Use syringe to measure out Di-*n*-butyl phtalate, add resin to mixture and stir again. Use syringe to measure out Methyl nadic anhydrate and BDMA, add to the mixture and stir very thoroughly for 5 minutes.

Nerve sample dehydration and epoxy resin embedding protocol

Solution	Wash Time	Repeat Washes
15% ethanol	5 mins	2
30% ethanol	10 mins	2
50% ethanol	15 mins	2
70% ethanol	30 mins	2
Dehydrated ethanol	20 mins	3
Dehydrated ethanol	60 mins	3
1,2 epoxy propane	15 mins	3
Epoxy resin + 1,2 epoxy propane 1:1	60 mins	1
Epoxy resin + 1,2 epoxy propane 3:1	Overnight	1
Epoxy resin	Overnight	1
Embed in epoxy resin	24 hours at 66°C	-

Preparation of stains for semi-thin epoxy resin sections

Thionin	
Thionin	0.5g
Distilled water	100ml
90% industrial methylated spirit (IMS)	125ml
0.1M NaOH	25ml
Add together in above order and heat to 50°C; filter. Cool and store in refrigerator.	
Acridine orange	
Acridine orange	2.5g
Distilled water	62.5ml
0.1M NaOH	62.5ml
Add together, heat gently, stir well and filter. Store in a refrigerator.	

Appendix 3 - Paraffin embedding of sections

Frozen muscle blocks up to $2cm^2$ were placed into 10% buffered formalin and fixed for 24 hours.

Processing was then undertaken using a routine 16 hour overnight schedule on a Tissue VIP machine (Miles Scientific, UK), with samples being embedded in BDH Gurr paramat wax (BDH Chemicals, Poole, Dorset, UK).

4µm sections were cut and mounted on Snowcoat Extra slides (Surgipath, St. Neots, Cambs, UK) and dried at 60°C for 2 hours prior to IHC staining.

Subsequent dewaxing and dehydration of sections was undertaken using xylene and industrial methylated spirits (Genta Medical, York, UK).

Appendix 4 - Muscle fibre type staining

The 3 muscle fibre types (I, IIa and IIb) were demonstrated on one section using double staining, a labelled streptavidin biotin horseradish peroxidase (HRP), followed by a streptavidin biotin alkaline phosphatase technique.

4µm paraffin sections were dewaxed in xylene for 5 minutes, followed by rehydration in graded industrial methylated spirits (IMS) and then washed under running tap water for 5 minutes.

Sections were microwaved at full power (800w commercial microwave) in 500mls of TRIS EDTA buffer (pH 9.0) for 10 minutes, being left to stand in the hot buffer solution for a further 20 minutes.

Further antigen retrieval was performed with incubation of the sections in Trypsin solution (Trypsin ICN 150mg, 150mls Tris HCL buffer [pH7.6 at 37°C], 3mls 5% $CaCl_2$) for 1 minute. The sections were then washed under cold running tap water for several minutes.

Endogenous peroxidase was blocked by incubation in 3% H_2O_2 in methanol for 10 minutes, following which the sections were again washed under cold running tap water for 5 minutes.

The sections were then placed in Tris Buffer Solution (TBS) with 0.1% tween 20 for 2-3 minutes, this TBS/tween detergent was used for all subsequent washes.

The sections were then placed on a Sequenza IHC staining machine (Thermoshandon, UK) for all subsequent solutions.

Biotin blocking was performed using 100µls of Streptavidin D blocking solution (Streptavidin Biotin Blocking Kit SP2002, Vector Laboratories, Peterborough, UK) applied for 15 minutes, followed by rinsing in TBS/tween and the process repeated using the biotin blocking solution.

The sections were then incubated in 100µls of 20% normal horse serum (NHS) in Chemmate dilutent (Dako S2202, Dako, Ely, UK) for 30 minutes.

Sections were incubated in monoclonal antibody to slow myosin (NOQ 7.5.4D, Sigma Aldrich, Poole, Dorset, UK) diluted to 1:2000 for 72 hours at 4°C and then washed in TBS/tween for 10 minutes.

[Negative controls for each block were also processed with Chemmate dilutent being substituted for the primary antibody.]

Sections were incubated in 100µls of biotinylated horse anti-mouse antibody (Vector Laboratories, UK), diluted 1:100 with Chemmate dilutent, for 30 minutes followed by washing in TBS/tween for 10 minutes

Setions were incubated in 100µls of streptavidin HRP (Vector Laboratories, UK) diluted 1:200 in Chemmate dilutent for 30 minutes followed by further washing in TBS/tween for 10 minutes.

Vector SG substrate solution (Vector Laboratories, UK) was applied for 5 minutes and then reapplied for a further 5 minutes followed by washing with TBS/tween for 10 minutes.

Biotin blocking was performed using 100µls of Streptavidin D blocking solution (Streptavidin Biotin Blocking Kit SP2002, Vector Laboratories, Peterborough, UK) applied for 15 minutes, followed by rinsing in TBS/tween, application of the biotin blocking solution for 15 minutes and further rinsing with TBS/tween.

The sections were then incubated in 100µls of 20% normal horse serum (NHS) in Chemmate dilutent (Dako S2202, Dako, Ely, UK) for 10 minutes.

Sections were incubated in monoclonal antibody to fast myosin (MY 32, Sigm Aldrich, Poole, Dorset, UK) diluted 1:50 in Chemmate dilutent for 12 hours at 4°C followed by washing with TBS/tween for 10 minutes.

Sections were incubated in 100µls of biotinylated horse anti-mouse antibody (Vector Laboratories, UK), diluted 1:100 with Chemmate dilutent, for 30 minutes followed by washing in TBS/tween for 10 minutes.

Sections were incubated in 100µls of streptavidin alkaline phosphatase (Vector Laboratories, UK) diluted 1:200 in Chemmate dilutent for 30 minutes and washed in TBS/tween for 10 minutes.

The sections were then incubated in Vector red alkaline phosphatase substrate solution (Vector Red SK 5100, Vector Laboratories, UK) for 5 minutes, the process then being repeated.

The sections were then washed in TBS/tween for 5 minutesand removed from the Sequenza and washed under running tap water for 5 minutes. The cell nuclei were counterstained using Harris' haematoxylin (applied for 20 seconds), the sections then being dehydrated through graded IMS, cleared inxylene and mounted in synthetic medium (DXP, BDH Chemicals, Poole, Dorset, UK).

Appendix 5 - PGP 9.5 nerve staining

4µm paraffin sections were dewaxed in xylene for 5 minutes, followed by rehydration in graded industrial methylated spirits (IMS) and then washed under running tap water for 5 minutes.

Sections were microwaved at full power (800w commercial microwave) in 500mls of TRIS EDTA buffer (pH 9.0) for 15 minutes, being left to stand in the hot buffer solution for a further 15 minutes.

Endogenous peroxidase was blocked by incubation in 3% H_2O_2 in methanol for 10 minutes, following which the sections were again washed under cold running tap water for 5 minutes.

The sections were then placed in Tris Buffer Solution (TBS) with 0.1% tween 20 for 2-3 minutes, this TBS/tween detergent was used for all subsequent washes.

The sections were then placed on a Sequenza IHC staining machine (Thermoshandon, UK) for all subsequent solutions.

Biotin blocking was performed using 100µls of Streptavidin D blocking solution (Streptavidin Biotin Blocking Kit SP2002, Vector Laboratories, Peterborough, UK) applied for 15 minutes, followed by rinsing in TBS/tween and the process repeated using the biotin blocking solution.

The sections were then incubated in 100µls of 20% normal horse serum (NHS) in Chemmate dilutent (Dako S2202, Dako, Ely, UK) for 30 minutes.

Sections were washed in TBS/tween, followed by application of 100µls 1:5 normal horse serum diluted in Chemmate dilutent for 30 minutes.

PGP 9.5 primary antibody (diluted 1:100 in Chemmate dilutent) was applied to the sections for 30 minutes followed by washing with TBS/tween for 10 minutes.

[Negative controls for each block were also processed with Chemmate dilutent being substituted for the primary antibody.]

Sections were incubated in 100µls of biotinylated horse anti-mouse antibody (Vector Laboratories, UK), diluted 1:100 with Chemmate dilutent, for 15 minutes followed by washing in TBS/tween for 10 minutes

Sections were incubated in 100µls of ABC solution (CSA Kit, K1500,Dako, Ely, UK - subsequent reagents all taken from this kit unless stated otherwise) for 15 minutes followed by further washing in TBS/tween for 5 minutes.

Sections were then treated with 100µls of tyramine amplification solution for 10-15 minutes and then washed in TBS/tween for 5 minutes.

Sections were then treated with 100µls of streptavidin-peroxidase reagent for 15 minutes and then washed with TBS/tween for 5 minutes.

Vector VIP substrate chromogen (Vector Laboratories, Peterborough, UK) was applied for 5 minutes, with staining assessed using a microscope, the slide being washed (with TBS/tween for 5 minutes and then under cold running tap water for 5 minutes) once adequate staining had been achieved.

Sections were counterstained with a 1% light green counterstain for 1 minute, washed under cold running tap water and then dehydreated using graded IMS, cleared in xylene and mounted in synthetic medium (DXP, BDH Chemicals, Poole, Dorset, UK).

Appendix 6 - NCAM muscle staining

4µm paraffin sections were dewaxed in xylene for 5 minutes, followed by rehydration in graded industrial methylated spirits (IMS) and then washed under running tap water for 5 minutes.

Sections were microwaved at full power (800w commercial microwave) in 500mls of TRIS EDTA buffer (pH 9.0) for 15 minutes, being left to stand in the hot buffer solution for a further 15 minutes.

The sections were then placed in Tris Buffer Solution (TBS) with 0.1% tween 20 for 2-3 minutes, this TBS/tween detergent was used for all subsequent washes.

The sections were then placed on a Sequenza IHC staining machine (Thermoshandon, UK) for all subsequent solutions.

Biotin blocking was performed using 100µls of Streptavidin D blocking solution (Streptavidin Biotin Blocking Kit SP2002, Vector Laboratories, Peterborough, UK) applied for 15 minutes, followed by rinsing in TBS/tween and the process repeated using the biotin blocking solution.

The sections were then incubated in 100µls of 20% normal horse serum (NHS) in Chemmate dilutent (Dako S2202, Dako, Ely, UK) for 30 minutes.

Sections were washed in TBS/tween, followed by application of 100µls 1:5 normal horse serum diluted in Chemmate dilutent for 30 minutes.

NCAM primary antibody (NCL CD 56.504, Novacastra Laboratories, UK) (diluted 1:60 in Chemmate dilutent) was applied to the sections for 12 hours at 4°C followed by washing with TBS/tween for 10 minutes.

[Negative controls for each block were also processed with Chemmate dilutent being substituted for the primary antibody.]

Sections were incubated in 100µls of biotinylated horse anti-mouse antibody (Vector Laboratories, UK), diluted 1:100 with Chemmate dilutent, for 30 minutes followed by washing in TBS/tween for 10 minutes

Sections were incubated in 100µls of streptavidin alkaline phospatase diluted 1:200 with Chemmate dilutent for 30 minutes and then washed with TBS/tween for 5 minutes.

Sections were incubated with Vector red substrate (made up in 200mMol Tris HCL pH 8.2) (Vector Laboratories, UK) for 5 minutes, the process then being repeated.

Nuclei were counterstained with Harris' haematoxylin for 30 seconds and the muscle fibres stained with 1% light green in 1% acetic acid for 1 minute. They were then washed under cold running tap water and then dehydrated using graded IMS, cleared in xylene and mounted in synthetic medium (DXP, BDH Chemicals, Poole, Dorset, UK).